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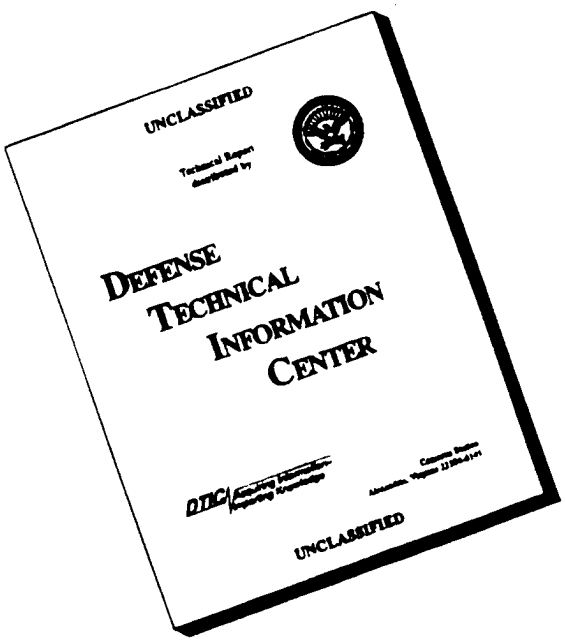

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13. ABSTRACT *(Maximum 200 words)*

Human progesterone receptor (hPR) in T47D breast cancer cells is phosphorylated upon hormonal treatment. The phosphorylation includes a rapid ^{32}P incorporation into hPR followed by a characteristic decrease of hPR mobility on SDS gel electrophoresis (SDS-PAGE) without further significant increase of phosphorylation. Current studies have shown that hPR can be activated by the antiprogestin RU486 in the presence of 8-Br cAMP. This switch of RU486 antagonist activity to agonist activity is thought to related to the phosphorylation of hPR or factors associating with hPR. To understand the role of phosphorylation in hPR activity and the switch, I sought to locate the phosphorylation sites in hPR. Thus far, I have identified eight phosphorylation sites including three hormone-dependent phosphorylation sites, Ser^{102}, Ser^{294} and Ser^{345} and five basal phosphorylation sites, Ser^{81}, Ser^{162}, Ser^{190}, Ser^{294} and Ser^{548}. Of these sites, Ser^{81}, Ser^{102} and Ser^{162} are in the PR-B specific region. Interestingly, phosphorylation of Ser^{345} is required for the altered mobility on SDS-gel electrophoresis. In addition, I have found that PR-B but not PR-A is responsible for the switch. Finally, I have shown that mutation of Ser^{81}, a CKII site, to Ala dramatically decreased the transcriptional activity of the receptor using a transient transcription assay.

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Yoram Vodovotz 7/24/95

PI - Signature Date

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I. Introduction

Breast cancer is the second leading disease that causes the death of American women. Despite extensive research efforts, the causes of breast cancer remain largely unknown; the current means of prevention and therapies are not entirely effective. At present, the major endocrine therapy for breast cancer utilizes the antiestrogen tamoxifen, and antiprogestin RU486 (1,2). Since the effectiveness of endocrine treatment requires functional receptors, it is crucial to determine what regulates the activity of the receptors. This study is designed to understand the role of phosphorylation in modulating human progesterone receptor (hPR) function.

hPR is a member of the steroid/thyroid hormone receptor family of transcription factors (3). It consists of two natural occurring forms, PR-A Mr: 97k Da and PR-B Mr: 120k Da (4,5). Like other members of the family, hPR contains a highly conserved DNA binding domain, a ligand binding domain, and a variable N-terminal region, which is a transactivation domain (AF-1). There is also a hormone dependent activation domain (AF-2) in the carboxyl terminus.

hPR is highly phosphorylated in T47D breast cancer cells upon hormonal stimulation (6,7). The rapid phosphorylation takes place in a few minutes after the addition of hormone. Prolonged treatment up to 60 min does not change the overall ^{32}P incorporation significantly, but decreases the mobility of hPR (upshift) on SDS gel electrophoresis (SDS-PAGE), indicating that phosphorylation can cause significant conformational changes within hPR.

Phosphorylation is a key process that can modulate functions of many proteins. Several lines of evidence have shown that phosphorylation of steroid receptors affects the binding of receptor to hormone onto DNA (6,8,9), and receptor nucleocytoplasmic shuttling (10). However, in vivo functional analysis remains incomplete and only a few studies have been documented. Until now, our lab has identified four phosphoserines in chicken PR (cPR) (11,12). Mutation of the hinge Ser^{530} phosphorylation site (between the DNA-and ligand-binding domain) to Ala decreases hormone sensitivity, implying that phosphorylation of this site is important for transcriptional activity at physiological levels of hormone (13). In addition, mutation of Ser^{211} to Ala reduced transcriptional activity of cPR dramatically (Bai and Weigel, in prep). Western analysis of the Ser^{211} mutant showed that the level of upshift was reduced, inferring that phosphorylation of Ser^{211} affects the conformation of cPR. Significant reduction of transcriptional activity was also reported for an estrogen receptor mutant (14). In contrast, initial studies on mutant glucocorticoid receptor (GR) suggested that although phosphorylation affected the stability of GR, effect on transcriptional activity was insignificant (15). However, more recent studies on GR mutants have shown that certain phosphorylation sites when mutated do affect receptor function (personal communications, K. Yamamoto, P. Housley).

The important role of phosphorylation in regulating receptor function is also supported by a striking observation that antagonist activity of RU486 can be switched to agonist activity in the presence of protein kinase A activator, 8-Br cAMP, in T47D cells (16,17). Similar results were also found for GR using RU486 (18) and ER using the antiestrogen tamoxifen (19). These finding are particularly important for the use of antihormones for breast cancer and may explain in part the cancer resistance to endocrine therapy. Whether it is altered phosphorylation of hPR or

factors associating with hPR that causes the switch remains as an interesting question, and thus to find out the mechanism becomes a central piece of my proposal.

Previous study of phosphorylation of hPR showed that hPR contains multiple phosphoserines located predominantly in the N-terminal region (7). Early evidence also indicated that there are several sites located in the B specific region (20). Phosphorylation of B specific sites are of particular interest since several studies have shown that PR-A and PR-B function differently in a cell type and promoter dependent manner. More interestingly, PR-A has been demonstrated to repress activities of PR-B and other steroid receptors such as androgen receptor, GR, mineralocorticoid receptor, and estrogen receptor (ER) (21,22). Why the function of PR-A is so distinct from that of PR-B, however, is not clear. Since PR-A and PR-B differ only in 164 amino acids at N-terminal, it is possible that the B specific region determines the activities unique to PR-B. Recently, Sartorius et al. (23) demonstrated that the N-terminal region unique to PR-B acts as a third transactivation domain in addition to AF-1 and AF-2. Furthermore, they showed that this domain alone is responsible for the band heterogeneity observed on SDS-PAGE for full length PR-B, suggesting that phosphorylation of B specific sites is responsible. Our report on the identification of two B specific sites strongly supported this notion (24). Detailed mutational analysis of hPR in the future will not only help us to understand the cause of heterogeneity but also to gain more understanding of the differential activity of PR-A and PR-B. More recently, Sartorius et al. (17) reported that the RU486 antagonist to agonist switch is specific for PR-B. We have reached the same conclusion using transient transfection assays. Although other mechanisms cannot be ruled out, we think phosphorylation of B specific sites or factors that interact with B specific region may be essential for the switch. Therefore, a thorough examination of phosphorylation of hPR in the presence of RU486 and 8-Br cAMP is necessary.

In order to understand the role of phosphorylation in hPR function, I decided first to identify all of the sites, focusing on agonist or antagonist dependent sites as well as sites altered by cotreatment with RU486 and 8-Br cAMP. Additionally, I would investigate the mechanism that converts RU486 from an antagonist to an agonist in the presence of 8-Br cAMP. Finally, for all the sites identified, I would perform site-directed mutagenesis on hPR and examine roles of these mutants with respect to their biological functions.

II. Body

A. Materials and Methods

Materials

Minimum essential medium (MEM) was purchased from Irvine (Santa Ana, CA). Phosphate-free MEM was obtained from GIBCO BRL (Grand Island, NY). AB-52 antibody that recognized both PR-A and PR-B is produced from Mouse monoclonal immunoglobulin G. R5020 and carrier-free [^{32}P]H_3PO_4 were puchased from Dupont/New England Nuclear Products (Boston, MA). Protein-A Sepharose was purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Tosylphenylalanyl chloromethy ketone-treated trypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ). Sequencing grade endoproteinases Asp-N and Glu-C were purchased from Boehringer Mannheim (Indianapolis, IN). Phenylisothiocyanate and sequencing grade trifluoroacetic acid (TFA) and HPLC reagents were purchased from J. T. Baker Chemical Corp. (Phillipsburg, NJ). Triethylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) was purchased from Sigma (St. Louis, MO). Sequelon-AA membranes and Mylar sheets were obtained from Millipore Corp. (Milford, MA). Transformer^{TM} site-directed mutagenesis kit was obtained from Clontech (Palo Alto, CA). Sequenase version 2.0 DNA sequencing kit was obtained from USB (Cleveland, Ohio). All other chemicals were reagent grade.

Cell Culture, PR labeling, and Receptor Preparation T47D human breast cancer cells were maintained and grown in 75-cm^2 T-flasks with frequent changes of media as previously described (24). Cells were incubated for 24 h in MEM containing 5% fetal calf serum that has been stripped of steroid hormones by dextran-coated charcoal treatment. Steady state labeling with [^{32}P]orthophosphate was carried out in phosphate-free serum-free medium for 1 h at 37^{0}C and then incubated in phosphate-free MEM containing [^{32}P]orthophosphate (0.83 mci/ml) for 6 h at 37^{0}C. Cells were treated with 40 nM R5020 for 2 h before harvest.

Cells were harvested in 1 mM EDTA in Earle's balanced salt solution and homogenized at 4^{0}C in a Teflon-glass Potter-Elvenhjem homogenizer (Fisher, Pittsburgh, PA) in KPFM buffer [50 mM potassium phosphate (pH 7.4), 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 12 mM monothioglycerol] containing 0.5 M NaCl and a mixture of proteinase inhibitors as previously described (24). The whole cell extract was obtained by centrifuging the homogenates at 100,000 x g for 30 min, and followed by dialyzed in KPFM to remove the salt before the immunoprecipitation step.

Immunoprecipitation and Gel Purification of PR Monoclonal antibody, AB-52 bound Protein-A Sepharose was prepared as previously described. Dialyzed whole-cell extracts containing PR (24) were incubated with Protein-A Sepharose on an end-over-end rotator for 4 h at 4^0 C. Protein-A Sepharose was washed at least three times with buffer KPFM containing 0.3 M NaCl to remove nonspecific proteins. Bound receptors were then eluted with 2% SDS sample buffer and electrophoresed on a 7.0% discontinuous SDS polyacrylamide gel. ^{32}P-labeled receptors were located by autoradiography of the gels, and PR-A and PR-B were retrieved by excising the corresponding gel pieces.

HPLC Analysis of Tryptic Peptides of PR The gel slices containing PR were washed with 50% methanol for 1 h followed by H_2O for 30 min and 50 mM ammonium bicarbonate for 5 min in a 1.5 -ml microfuge tubes. 20 ug trypsin was added to the tube. After incubating the tube for 4 h at 37^0C, another 20 ul trypsin was added, and this was repeated for 3 more times. The digested peptides were dried in a Speedvac (Savant Instruments, Hicksville, NY), dissolved in 150 ul 50% formic acid, loaded on a Vydac (Hesperia, CA) C18 revese phase column in 0.1% TFA in water, run at a flow rate of 1 ml/min, and eluted with a linear gradient from 0-45% acetonitrile over 90 min. The labeled peptides were detected with an on-line model IC Flo-One Beta-radioactivity flow detector (Radiomatic Instruments, Inc., Tampa, FL), and collected as 1 ml fractions.

Phosphorylation Site Identification Fractions corresponding to each labeled peptide were dried and further separated by electrophoresis on a 40% alkaline gel. Labeled peptides were detected by autoradiography the of dried gel, excised, and eluted with H_2O as previously described (24).

To find the position of phosphoamino acids in the peptides, I used manual Edman degradation as described by Sullivan and Wong (25). In brief, the peptide to be analyzed was dissolved in 30 ul of 50% acetonitrile, and spotted on an arylamine-Sequelon disc, which was placed on a Mylar sheet on top of a heating block set at 50°C. After 5 min, the aqueous solvent was evaporated and the disc was removed from the heating block. 5 ul of EDAC solution (50 mM in Mes, pH 5.0) was added to the disc to allow the peptide to covalently link to the disc, and the disc was placed at RT for 30 min. The disc was then washed 5 times with water and 5 times with TFA to remove unbound peptide. The disc was then washed 3 times with methanol, and subjected to Edman degradation: The disc was treated at 50°C for 10 min with 0.5 ml coupling reagent (methanol:water:triethylamine:phenylisothiocyanate; 7:1:1:1, v/v). After 5 washes with 1 ml of methanol, the disc was treated at 50°C for 6 min with 0.5 ml TFA to cleave the amino terminal amino acid. The TFA solution was placed in a scintillation vial and the disc was washed with 1 ml of TFA and 42.5% phosphoric acid (9:1, v/v). The wash was combined with the TFA solution and the released [^{32}P] was determined by Cerenkov counting. The next cycle began after the disc was washed 5 times with 1 ml methanol.

To characterize the peptides, the tryptic peptides were digested with the endoproteinases Glu-C and Asp-N. Glu-C cuts on the C-terminal side of Glu, except for Glu-Pro bonds. Moreover, Glu-X bonds within three residues of the end of a peptide are cleaved poorly (26). Asp-N cuts on the N-terminal side of Asp residues. Peptides, digested and undigested, were loaded to a peptide gel electrophoresis or manual Edman degradation. Glu-C digestion was performed in 200 ul 25 mM ammonium bicarbonate, pH 7.8, for 8 h at 37^0C. Asp-N digestion was perfomed in 200 ul 50 mM sodium phosphate buffer, pH 8, containing 0.2 ug Asp-N and incubated at 37^0C for 4 h.

Preparation and Isolation of Peptides for Sequencing Purified Baculovirus-expressed PR-B (supplied by Dr. Dean Edwards' lab) was digested with trypsin (5% wt/wt). Tryptic peptides were separated by reverse phase HPLC as described earlier. Fractions with retention times corresponding to the ^{32}P-labeled tryptic phosphopeptides from T47D cells were collected, dried and sequenced using an automated sequencer (11).

Site-directed Mutagenesis and Seqencing Site-directed mutagenesis was perfomed using a kit from Clontech. Briefly, Two primers were simultaneously annealed to one strand of the denatured double-stranded expression vector. The selection primer contains a mutation which changes the restriction site from Nde 1 to Afl II in the plasmid backbone. The mutagenesis primer changes Ser to Ala. After DNA elongation, ligation and a primary selection by digesting with restriction enzyme Nde 1, the mixture of mutated and unmutated plasmids were transformed into a mutS E. coli strain defective in mismatch repair. Plasmid DNA was enriched from the pool of transformants and and digested again with Nde 1 and transformed in DH5α bacterial strain. Transformants resistant to Nde 1 digestion were selected and plasmid DNA prepared and sequenced using a USB Sequenase Version 2.0 DNA Sequencing Kit based on chain-termination sequencing theory (27).

Transfection and CAT Assays CV1 cells (monkey kidney) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin at 100 ug/ml, and streptomycin at 100 ug/ml in a humidified atmosphere of 5% CO_2 and 95% air. Twenty four hours before transfection, 1 x 10^6 cells were plated in 10-cm dishes in DMEM supplemented with 10% fetal bovine serum and were allowed to attach. The cells were washed with Hanks' Balanced Salt Solution (HBSS) lacking calcium and magnesium and then incubated in DMEM supplemented with 1% Nutridoma SR. Before transfection, cells were washed once with HBSS and incubated in 10 ml serum-free DMEM media. Five ug reporter and various amount of receptor DNA in 1 ml HBS (1mM $Na_2HPO_4.7H_2O$, 23 mM HEPES, pH 7.05) were mixed with polybrene (50 ug) and added dropwise to the cell culture and incubated with cells for 3 to 4 h. Cells were then treated with 25% glycerol in HBSS for 30 sec, washed twice with HBSS, and grown in media containing Nutridoma SR for another 48 h. Hormones were added 18 h after the glycrol shock.

Each plate of cells was washed once with PBS without calcium and magnesium, scraped into 1 ml TEN buffer (40 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 8.0) and collected by centrifugation at 14,000 rpm for 10 sec. Cells were resuspended in buffer A (15 mM NaCl, 2 mM EDTA, 0.15 mM spermine, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride) and lysed by three freeze/thaw cycles. Protein concentration were determined by micro-plate Bradford assay. CAT activity was determined by incubating 25 ug protein with 0.2 uci [^{3}H]chloramphenicol (20 uci/umol) and 250 uM butyryl-coenzyme A in 100 ul 100 mM Tris-HCl, pH 8, for 2 h at 37^{0}C. Acylated chloramphenicol was extracted using a mixture of 200 ul 2:1 TMPD and Xylenes and counted in a scintillation counter. Values for reagent blanks are subtracted from all samples before calculation of data.

B. Results

1. Identification of phosphorylation sites in human PR. Over the years, in Dr. Weigel's lab, we have developed a strategy applicable to identifying phosphorylation sites in virtually any phosphoprotein with known sequence. The strategy includes the use of tryptic digestion, HPLC reverse phase chromatography, 40% alkaline gel, specific protease digestion, a baculovirus overexpression system, manual Edman degradation, and automated sequencing, which have been described in detail in the methods section. The procedure requires a minimum amount of ^{32}P labeled protein and identifies phosphorylation sites effectively and unambiguously. To date, in collaboration with Dr. Edwards's lab, I have identified eight phosphorylation sites. Three sites Ser^{81}, Ser^{102} and Ser^{162} are PR-B specific (24,28). The other sites identified are Ser^{190}, Ser^{294}, Ser^{345}, Ser^{400} and Ser^{558}. Ser^{102}, Ser^{345} and Ser^{294} are hormone inducible sites(28). All sites, except for $Ser^{81}$and Ser^{558}, are within Ser-Pro motifs. In addition, Ser^{345} appears responsible for the altered mobility on SDS gels based on the fact that the upshift of hPR on SDS gels was associated only with the phosphorylation of Ser^{345} (28). The results of the identification of three B specific sites and three hormone inducible sites were reported in detail in our recent publications (24,28), which are included in appendix. In this report, I will focus only on some new data.

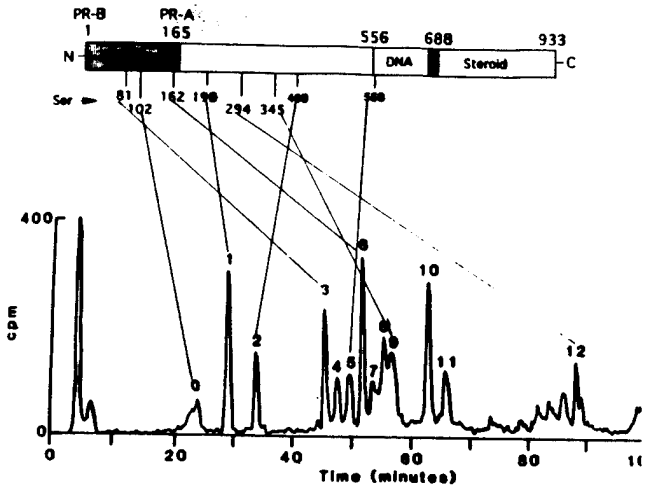

Figure 1. Location of phosphorylation sites in human progesterone receptor

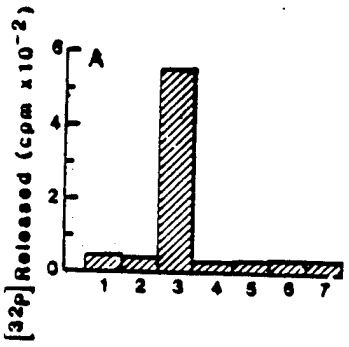

Figure 2. Phosphate release analysis of peptide 1

Identification of Ser^{190} as a phosphorylation site

Fig. 1 illustrates a map of phosphotryptic peptides of hPR phosphorylated in vivo in the presence of hormone and a diagram of hPR structure. The location of phosphorylated sites and peptides containing the sites are indicated by lines that connect both.Each phosphopeptide has been numbered based on the order of its retention time. In order to identify phosphorylation site in peptide 1 (p1), HPLC fractions containing p1 were subjected to specific proteinase digestion with Asp-N and Glu-C, which cut, respectively, at the N-terminal side of Asp residues and the C-terminal side of Glu residues. The digested peptide was subjected to a 40% alkaline polyacrylamide gel, which is capable of resolving small peptides to determine if it contains a cleavable Asp or Glu based on a change in mobility. p1 cannot be cleaved by either Asp-N or Glu-

C since digested and undigested peptides have the same mobility on the gel (data not shown), suggesting the absence of both Asp and Glu in p1. Manual Edman degradation experiment determined that the phosphorylated residue is located at the third position in p1 as shown in Fig.2. Since all the phosphorylation sites in hPR are Ser (29), I listed in table 1 all the potential candidates containing Ser in position 3. It appears that peptide containing Ser^{190} is the only candidate that fits all the criteria. In order to confirm the prediction, a phosphopeptide GLSPAR was synthesized and loaded to the HPLC along with in vivo ^{32}P labeled p1 as a marker. Fraction containing the ^{32}P labeled p1 was collected, dried, further isolated by a 40% alkaline gel and autoradiographed. The band corresponding to p1 was excised and the peptide eluted with water. The purified peptide was subjected to automated sequencing, which showed a single peptide sequence as illustrated in table 2. Therefore, we have confirmed that Ser^{190} is phosphorylated in vivo.

TABLE 1

Potential Tryptic Phosphopeptide Candidates for Peptide 1

The number preceding the peptide indicates the position of the first amino acid of the peptide within the sequence of PR-B

Phosphoserine in Third Position

168	VLSPLMSR
188	GLSPAR
271	EDSR
547	PDSEASQSPQYSFESLPQK
770	HVSGQMLYFAPDLILNEQR
791	ESSFYSLCLTMWQIPQEFVK
900	ALSVEFPEMMSEVIAAQLPK

Identification of Ser^{400}

Peptide 2 (p2) can be cleaved by Glu-C but not Asp-N as shown in Fig.3. The manual Edman degradation suggests that phosphoserine is at amino acid 14 and at cycle 5 after peptide 2 is digested by Glu-C (Fig. 4). However, none of the tryptic peptides containing Ser in cycle 14 can be cleaved by Glu-C to produce a peptide with Ser in cycle 5. To verify whether p2 is a result of incompleted digestion, it was treated with trypsin. The redigestion with trypsin in Fig, 3 suggests tht peptide 2 contains more than one tryptic peptide and that the authentic tryptic peptide is a small peptide which may not bind to the HPLC column. Therefore, the drop through was collected and manual Edman degradation performed. It turned out that majority of the ^{32}P released on cycle 1, suggesting that there is a small peptide in the drop through. Based on the information from release and protease digestion, we believe that peptide 2 is a product of partial tryptic digestion. The predicted sequence, partial and completely digested products are listed in table 3. We also used baculovirus expressed PR to confirm the analysis. Baculovirus PR was digested with trypsin (5% trypsin to receptor, wt/wt). The tryptic peptides were separated by HPLC. The fraction corresponding to ^{32}P labeled phosphopeptide 2 was collected and subjected to automated sequencing. The sequencing

Table 2

Peptide Sequence Analysis

Peptides 1 was sequenced as described in the text

Cycle	Amino acid	Position	pmol
Peptide 1 Sequence			
1	Gly	188	1354
2	Leu	189	1058
3	Ser	190	76
4	Pro	191	671
5	Ala	192	973
6	Arg	193	323

Figure 3. Characterization of phosphopeptide 2 by redigestion with additional proteases

data reveals the first 10 amino acids of our predicted peptide beginning with Ile^{387} as shown in table 4. Evidently, Ser^{400} is the phosphorylated site.

Identification of Ser^{558}

Fig. 5 shows that peptide 5 (p5) contains a phosphorylated Ser at position 12. As shown in Table 5, there are three peptides containing Ser at position 12. The peptide starting with Pro31 is eliminated immediately for it is a B specific peptide. I next perfomed Asp-N and Glu-C digestion followed by peptide gel separation. Fig. 6 shows that p5 cannot be cleaved by Asp-N; however, Glu-C digested p5 shows two bands on the film indicating multiple digestable Glu residues in p5. The peptide starting with Pro^{434} contains only one cleavable Glu, hence the peptide starting with Pro^{547} is a better candidate for p5. In addition, according to relative retention time predicted based on the hydrophobicity of each peptide, the peptide starting with Pro^{434} should be eluted after peptide 12 while the peptide starting with Pro^{547} is predicted to be eluted in between p3 and p6. Therefore, Ser^{558} is identified as a phosphorylation site.

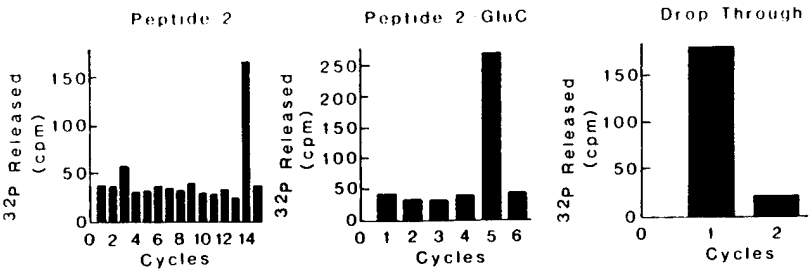

Figure 4. Identification of the cycle containing phosphoserine by Manual Edman Degradation

Table 3

Result of proteases digestion of phosphopeptide 2

Peptide 2	387 I K E E E E G A E A S A R S P R
Glu-C treated	396 A S A R S P R
Trypsin treated	400 S P R

Table 4

Peptide Sequence Analysis

Peptides 2 was sequenced as described in the text

Cycle	Amino acid	Position	pmol
Peptide 1 Sequence			
1	Ile	387	32
2	Lys	388	19
3	Glu	389	16
4	Glu	390	17
5	Glu	391	13
6	Glu	392	14
7	Gly	393	21
8	Ala	394	18
9	Glu	395	9
10	Ala	396	16

2. Any new sites in PR in the presence of RU 486 and 8-Br cAMP?

To test if 8-Br cAMP plus RU486 may generate new site(s) on PR-B, we have performed phosphopeptide mapping studies of hPR in T47D the same way as described in the methods in the presence of RU486 alone and RU486 plus 8-Br cAMP. The experimental data is included in appendix. We have not detected any differences in terms of phosphopeptide maps, suggesting that 8-Br cAMP treatment does not cause altered receptor phosphorylation. This result leads us to believe that there are factors under the control of PKA initiated signaling pathway (s) that interact

with the PR-B specific region resulting in the switch. Moreover, phosphorylation of B specific sites may play a role in interaction with other factors, and we will test this possibility with all the B mutants once they are made. Alternatively since PR-B, but not PR-A is a strong activator, it is possible that the sites common to the receptors are important for this activity.

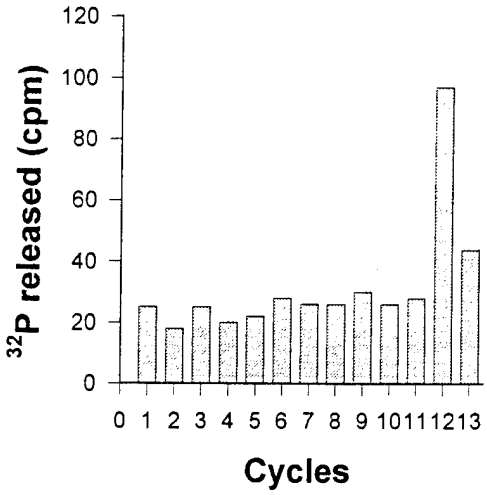

Figure 5. Phosphate release analysis of peptide 5

3. In vitro phosphorylation of human progesterone receptor

Since the majority of the sites identified are within Ser-Pro motifs, which is a core consensus for proline dependent kinases including Cdks and Map kinases, I next tested if Cdk2-cyclin A can phosphorylate hPR. Fig. 7 shows the phosphorylation of PR-A and PR-B. The percentage of phosphorylation is about 1 (N=4) suggesting that PR is a good substrate for Cdk2. Fig. 8 shows the tryptic map of in vitro phosphorylated PR-A and PR-B. The retention time of in vitro phosphorylated tryptic peptides are the same as these in vivo labeled ones, suggesting that Cdk2-cyclin A complex phosphorylated predominantly on p1, p2, and p6, which represent Ser^{190}, Ser^{400} and Ser^{162} respectively. P4 is an overdigested peptide of p6, which also contains Ser^{162} (data not shown).

TABLE 5

Potential Tryptic Phosphopeptide Candidates for Peptide 5

The number preceding the peptide indicates the position of the first amino acid of the peptide within the sequence of PR-B

Phosphoserine in Twelveth Position	
31	PAAGPFPGSQTSDTLPEVSAIPISLDGLLFPRVLSPLMSR
434	PGEAAVTAAPASASVSSASSSGSTLECILYK
547	PDSEASQSPQYSFESLPQK

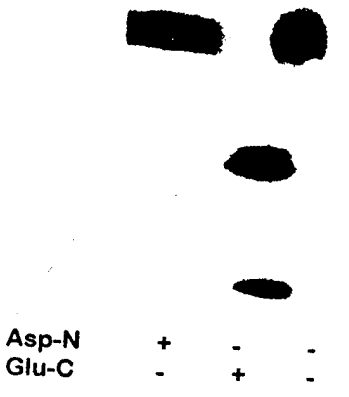

Figure 6. Characterization of peptide 5 by specific protease digestion

4. PR-B is responsible for the agonist activity of RU 486 in the presence of 8-Br cAMP

As I described earlier, PR-A and PR-B are structurally different and each may play different functional roles. Therefore, the ability of both PR-A and PR-B to elicit the switch was tested using plasmid expressing either PR-A or PR-B. Vectors expressing PR-A and PR-B were transfected to CV1 cells along with two different promoter driven CAT reporters using the method described earlier. The results are shown in Fig.(9) Consistent with other reports, PR-B but not PR-A was responsible for the switch in CV1 cells with reporters driven by two different promoters. PREtkCAT is driven by a complex promoter whereas $GRE_2E1bCAT$ is driven by a simple promoter containing a single TATA box. The greater swithch response

observed with PREtkCAT reporter is likely due to more factors interacting with the complex promoter.

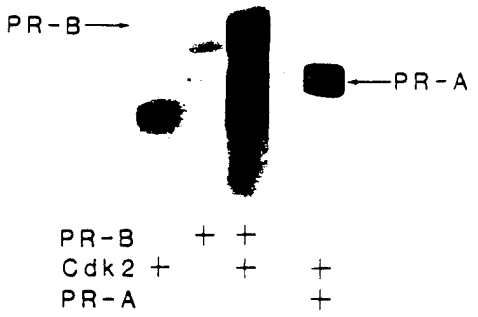

Figure 7. In vitro phosphorylation of PR by Cdk2

5. Site-directed mutagenesis and transcriptional activity of mutant PR

To assess the functional role of phosphorylation in vivo, we have just began to mutate sites that were identified, and to investigate the transcriptional activity of the mutants. Ser^{81} was mutated to Ala to mimic the unphosphorated state of receptor. The mutation from codon TCG to GCG was confirmed by chain-termination sequencing (27). The sequencing data is submitted as an appendix. Transcriptional activity of wt and mutant were tested by transfecting the plasmids into CV1 (monkey kidney cells) with a CAT reporter containing MMTV (mouse mammary tumor virus), and subsequently determining the CAT activity. I found that mutation of Ser^{81} to Ala significantly reduced the activity of MMTVCAT within the linear range, suggesting that phosphorylation of this site is very important for the transcriptional activity of hPR (Fig. 10). The activities of both wt and mutant were decreased at higher DNA concentration due to squelching effect. A top priority in the future will be the completion of the identification of the phosphorylation sites. Further characterization of the Ser^{81} mutant as well as of the B specific region as an activation domain will be carried out in the next year.

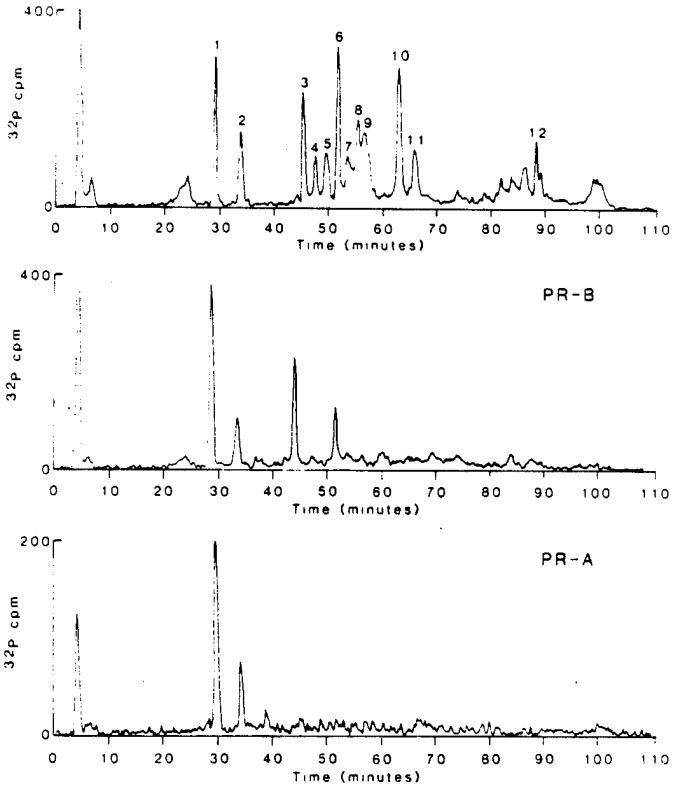

Figure 8. Phosphopeptide maps of PR phosphorylated in vitro with Cdk2

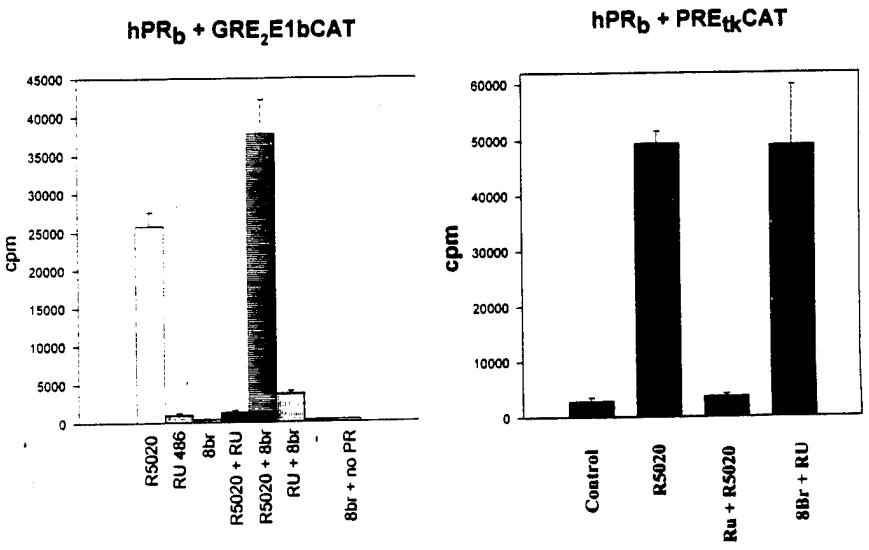

Figure 9. Transcriptional activity of PR-B in the presence of RU 486 and 8-Br cAMP in CV1 cells

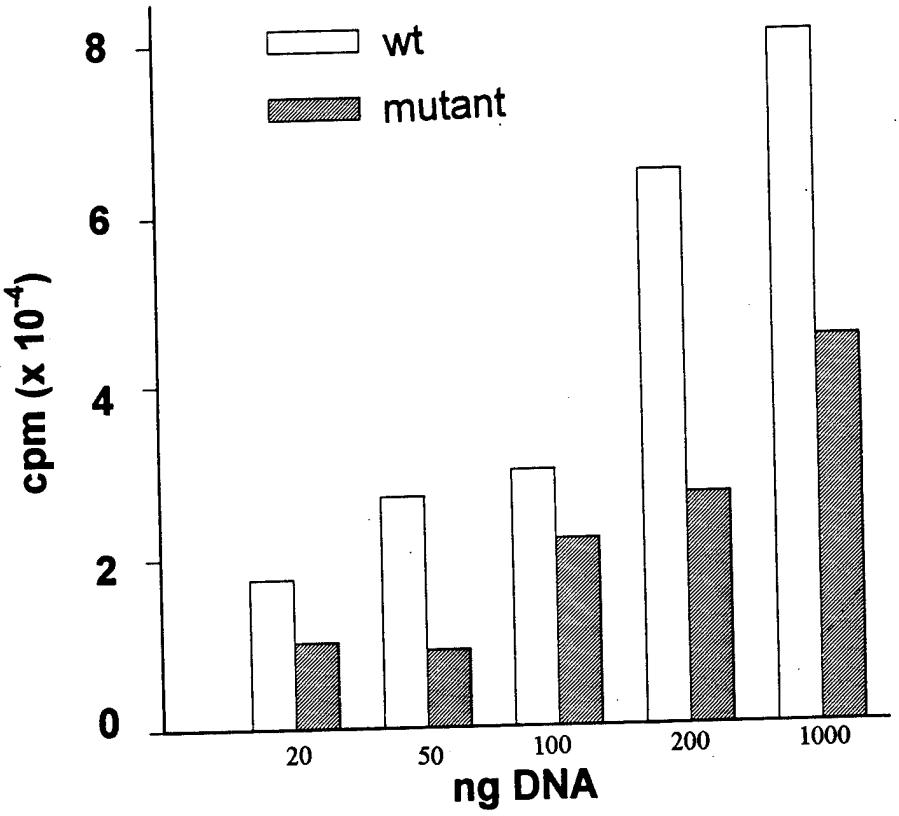

Figure 10. Comparison of the transcriptional activity of wild type and mutant Ala^{81} in CV1 cells

III Conclusions

The identification of multiple phosphorylation sites in hPR suggests that phosphorylation of hPR is very complex, and multiple protein kinases may be involved. Earlier, I demonstrated that Ser^{81} is within a CKII consensus, which appears to be a common site in steroid receptors (28). It is also common to steroid receptors that the majority of the phosphorylation sites are in a Ser-Pro motif, which is a target for proline-directed kinases including MAP kinases and cyclin-dependent kinases. This interesting phenomenon suggests that phosphorylation of hPR is perhaps downstream of MAP kinase pathways, and phosphorylation of hPR is associated with cell cycle.

The evidence that the switch is intrinsic to PR-B suggests that some factors may interact with the B specific region in the presence of both RU486 and 8-Br cAMP and thus in turn result in the activation of target genes. The finding is so important that to seek those factors and determine how they are regulated will be a significant step toward the understanding of the mechanism of the switch. Furthermore, the interaction may involve the phosphorylation of B specific sites. Therefore, with the identification of three B specific sites and mutants on those sites, we shall be able to test this hypothesis in the future.

As I mentioned earlier even though phosphorylation has been demonstrated to be important for many biological events, its role in steroid receptor function has not been clearly illustrated. Our initial functional studies with Ser^{81} mutant have provided strong evidence that phosphorylation plays an important role in receptor activity. My future studies will continue to enhance our knowledge of regulatory role of phosphorylation in steroid action.

In summary, I have accomplished more than what I proposed to do for the first year. The preliminary achievements will now help me to address some of the problems that could not be answered before. I will complete the identification of the sites, and seek to obtain initial information about the functional role of each individual phosphorylation site in hPR.

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Identification of Phosphorylation Sites Unique to the B Form of Human Progesterone Receptor

IN VITRO PHOSPHORYLATION BY CASEIN KINASE II*

(Received for publication, July 25, 1994)

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The human progesterone receptor (PR), a member of the steroid/thyroid receptor superfamily of ligand-activated transcription factors, is expressed in most tissues as two forms that exhibit differential transcriptional activation potentials, full-length PR-B and NH_2-terminally truncated PR-A. In human breast cancer cells (T47D) both forms of PR are constitutively phosphorylated but phosphorylation is increased in response to hormone treatment, suggesting that this modification has a role in regulating the activation state of the receptor. To more directly define the functional role of phosphorylation in the action of A and B receptors requires knowledge of the phosphorylated amino acid residues and the protein kinase(s) involved. Toward this end we have developed a strategy that combines isolation of PR phosphotryptic peptides by reverse phase high performance liquid chromatography, secondary analytical protease digestion, manual Edman degradation, and release of ^{32}P that resulted in identification of two major phosphorylation sites, Ser^{81} and Ser^{162}. Both sites are located in the amino-terminal region unique to PR-B, and one of these sites (Ser^{81}) is encompassed in a casein kinase II (CKII) consensus sequence. Although human PR contains 11 potential CKII consensus sequences, CKII *in vitro* phosphorylated purified PR-B only at Ser^{81} suggesting that this may be an authentic site for CKII *in vivo*.

Progesterone receptors (PR)[1] belong to a large family of ligand-activated transcription factors (1). PR in most species are expressed as two isoforms, PR-A and PR-B. Both forms are produced from a single gene either from separate messenger RNAs that arise by alternate use of two promoters or by initiation of translation from a second start site on a single PR mRNA (2–4). PR-A is an NH_2-terminally truncated version of PR-B.

PR in chicken oviduct (5, 6), rabbit uterus (7), and human breast cancer cells (8–10) are phosphorylated in the absence of hormone and undergo an increase in phosphorylation upon hormonal stimulation. Treatment of T47D human breast cancer cells with a progestin induces a 2-fold increase in net phosphorylation of human PR (hPR), and a characteristic decrease (or upshift) of receptor mobility on SDS-gel electrophoresis (11). A time course study under steady-state labeling conditions elucidated a two-phase phosphorylation mechanism; a rapid phosphorylation that occurs between 5 and 10 min after addition of hormone, accounting for most of the net increase, followed by the PR upshift that begins at 20 min and requires 40–60 min for completion. Interestingly, the phosphorylation associated with PR upshifts occurs with little additional change in net ^{32}P incorporation (11). Additional data have suggested that this late phase of phosphorylation may be both hormone- and DNA-dependent (12, 13).

It is becoming increasingly evident that phosphorylation of PR, as well as other steroid receptors, plays a role in regulating the activity of the receptors. Modulators of protein kinases and phosphatases such as 8-bromo-cAMP, okadaic acid, calyculin, vanadate, and epidermal growth factor have been used to assess the role of phosphorylation in regulating the activity of chicken PR (cPR) in transient transfection assays (14). These compounds are capable of activating receptor in the absence of hormone (14). This striking ligand-independent activation has also been reported for several other steroid receptors (15–18). In contrast to cPR, human PR does not appear to be susceptible to ligand-independent activation by modulators of protein phosphorylation. However, modulators such as cAMP, okadaic acid, and phorbol esters do potentiate hormone-dependent activation of human PR (11). Interestingly, the progesterone receptor antagonist RU486 can be converted to a partial but potent agonist by treating cells with 8-bromo-cAMP (19). Whether this antagonist to agonist switch is the consequence of altered receptor phosphorylation or phosphorylation of another protein involved in PR-mediated transcriptional enhancement remains to be determined.

There is increasing evidence that PR-A and PR-B have distinct functional properties that are dependent on the cell type and target genes with which they interact. PR-B when expressed alone in HeLa cells was reported to be able to mediate partial agonist activity of RU486 (20, 21), whereas PR-A alone was not capable of doing this. In addition, PR-A has been reported to be capable of exerting dual functional roles; to serve as a positive transcriptional activator or as a repressor (21, 22). In cells and target genes where PR-A exhibits no transcriptional activation properties, it has been reported to act as a repressor of PR-B-mediated transcription. Surprisingly, PR-A but not PR-B, can also act as a trans-repressor of glucocorticoid, androgen, and mineralocorticoid receptor-mediated transcription in a cell type and promoter-dependent manner (22). The mechanism for these distinct functional properties of PR-A and PR-B is not known. Clearly, the different NH_2-terminal se-

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[1] The abbreviations used are: PR, progesterone receptor; hPR, human PR; cPR, chicken PR; MEM, minimal essential medium; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CKII, casein kinase II; HPLC, high performance liquid chromatography; Mes, 4-morpholineethanesulfonic acid; MAP, mitogen-activated protein.

quences present in PR-A and PR-B must play a role in directing the distinct activities of the two PR isoforms.

Although phosphorylation is thought to have a regulatory function, the role of specific phosphorylation sites in PR function is still poorly understood. One of our laboratories has identified four phosphorylation sites in cPR (23, 24). All four sites are on serine residues that are common to both cPR_A and cPR_B. Two sites (Ser^{367} and Ser^{530}) are hormone-dependent phosphorylation sites. The phosphorylation of human PR is not as well characterized. Initial phosphopeptide mapping analysis by Sheridan *et al.* (8) showed that hPR phosphorylation is more complex than cPR. They reported that there are at least five constitutive phosphopeptides common to PR-A and PR-B and a sixth one is unique to PR-B. One additional phosphopeptide, and possibly a second, were detected only after hormone treatment. In addition, all phosphorylations are on serine residues. The exact location of hPR phosphorylation sites, however, has not been reported.

In the present study, we have identified two major phosphopeptides which are unique to the B form of hPR and have developed a simple strategy that should be applicable to other proteins and steroid receptors which requires a minimum amount of ^{32}P-labeled protein to identify phosphorylation sites. Using this strategy, we have identified Ser^{81} and Ser^{162} as the major PR-B specific phosphorylation sites. In addition, we have performed *in vitro* phosphorylation studies with casein kinase II (CKII) and purified PR-B showing that CKII preferentially phosphorylates Ser^{81}, which is part of a consensus sequence for CKII.

EXPERIMENTAL PROCEDURES

Materials—R5020 and carrier free $[^{32}P]H_3PO_4$ were obtained from DuPont-NEN. [γ-^{32}P]ATP was purchased from ICN (Irvine, VA). Protein A-Sepharose was obtained from Pharmacia Biotech Inc. Tosylphenylalanyl chloromethyl ketone-treated trypsin was obtained from Worthington. Sequencing grade endoproteinase Asp-N and Glu-C were purchased from Boehringer Mannheim. Phenylisothiocyanate and HPLC reagents were obtained from J. T. Baker Inc. Triéthylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), and sequencing-grade trifluoroacetic acid were obtained from Sigma. Sequelon-AA membranes and Mylar sheets were obtained from Millipore Corp. (Milford, MA). AB-52 is a mouse monoclonal immunoglobulin G (IgG) produced against purified human PR that recognizes both A and B forms of receptor (25). Casein kinase II was purchased from Promega (Madison, WI). Minimum essential medium (MEM) was purchased from Irvine (Santa Ana, CA). Phosphate-free MEM was obtained from Life Technologies, Inc. All other chemicals were reagent grade.

Purification of Baculovirus-expressed Human-PR-B—Human PR-B used in protein sequencing experiments and for *in vitro* phosphorylation was produced and purified as a full-length recombinant protein from the baculovirus expression system as described previously (26, 27). Purification was by single-step monoclonal antibody affinity chromatography using the B-30 antibody specific for PR-B (25). As judged by single-dimension silver stained SDS-gels and Western blot, PR-B preparations are routinely purified to apparent homogeneity (27). Receptors were bound to hormone (R5020) *in vivo* during the last 4 h of infection of *Spodaptera frugiperda* (Sf9) cells with the PR-B expressing recombinant virus. Thus purified PR used in these experiments was bound to hormone. Baculovirus produced hPR as reported previously is functionally indistinguishable from hPR synthesized endogenously in mammalian cells (26).

Cell Culture, Metabolic Labeling, and Receptor Preparations—T47D human breast cancer cells were cultured and grown for 2 weeks with frequent changes of media as described previously (11). Cells growing in 75-cm^2 T flasks (Falcon, Oxnard, CA) were incubated for 24 h prior to harvest with MEM containing 5% fetal calf serum that had been stripped of steroid hormones by treatment with dextran-coated charcoal. For steady-state labeling with [^{32}P]orthophosphate the serum-containing medium was removed and cells were preincubated in phosphate-free serum-free medium for 1 h at 37 °C. Cells were then incubated for 6 h at 37 °C in phosphate-free MEM containing [^{32}P]orthophosphate (0.83 mCi/ml). Cells were treated with 40 nM R5020 for times indicated in the figures.

Cells harvested with 1 mM EDTA in Earle's balanced salt solution were homogenized at 4 °C in a Teflon-glass Potter-Elvehjem homogenizer in KPFM buffer (50 mM potassium phosphate (pH 7.4), 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 12 mM monothioglycerol) containing 0.5 M NaCl and a mixture of proteinase inhibitors as described previously (11). The homogenates were centrifuged at 100,000 × *g* for 30 min, and the supernatant (whole cell extract) was dialyzed against KPFM to reduce salt concentration before immunoprecipitation.

Immunoprecipitation and Gel Purification of PR—Protein A-Sepharose was prebound with the receptor-specific monoclonal antibody, AB-52 (25) as described previously (11). Receptors in dialyzed whole cell extracts were then incubated with AB-52-coated protein A-Sepharose on an end over end rotator for 4 h at 4 °C. Protein A-Sepharose was washed repeatedly by centrifugation in buffer containing 0.3 M NaCl. Bound receptors were eluted with 2% SDS sample buffer and electrophoresed on 7.0% discontinuous SDS-polyacrylamide gels as described previously (11). ^{32}P-Labeled receptors were detected by autoradiography of wet gels and the gel pieces corresponding to the PR-A and PR-B isoforms were excised and incorporated radioactivity was measured by Cerenkov counting of the gel pieces.

HPLC Analysis of Trypsin-digested PR—SDS gel slices containing PR were cut and placed in 1.5-ml microcentrifuge tubes, washed with 50% methanol for 1 h, with H_2O for 30 min, and with 50 mM ammonium bicarbonate for 5 min. Twenty μg of trypsin were added to the tubes containing gel slices and 500 μl of 50 mM ammonium bicarbonate and incubated at 37 °C; four additional aliquots of trypsin were added at 1.5-h intervals. Tryptic phosphopeptides were dried and redissolved in 50% formic acid and applied to a Vydac C18 reverse phase column in 0.1% trifluoroacetic acid in water, run at a flow rate of 1 ml/min, and eluted with a linear gradient from 0 to 45% acetonitrile over 90 min. ^{32}P-Labeled peptides were identified on-line with a model IC Flo-One β radioactive flow detector (Radiomatic Instruments, Inc., Tampa, FL).(23).

Characterization of Tryptic Phosphopeptides by Proteinase Digestion and Manual ^{32}P Release—Fractions containing tryptic phosphopeptides resolved by reverse phase HPLC were further separated by electrophoresis on a 40% alkaline polyacrylamide gel (28). The gel was dried and autoradiographed. Bands containing tryptic phosphopeptides were excised and eluted with H_2O overnight, and dried in a Speedvac. Eluted peptides were subsequently subjected to digestion with the endoproteinases Glu-C and Asp-N which cut, respectively, on the COOH-terminal side of Glu and NH_2-terminal side of Asp residues followed by peptide gel electrophoresis or manual Edman degradation. Asp-N digestion was performed using 0.2 μg of Asp-N in 200 μl of 50 mM sodium phosphate buffer, pH 8.0, for 4 h at 37 °C. Glu-C digestion was performed using 1 μg of Glu-C in 200 μl of 25 mM ammonium bicarbonate, pH 7.8, for 8 h at 37 °C.

Manual Edman degradation studies were performed to localize the position of phosphoamino acids in the peptides using the method described by Sullivan and Wong (29). Briefly, the peptide to be analyzed was dissolved in 30 μl of 50% acetonitrile, and spotted on an arylamine-Sequelon disc, which was placed on a Mylar sheet on top of a heating block set at 50 °C. After 5 min, the aqueous solvent was evaporated, and the disc was removed from the heating block. 5 μl of EDAC solution (1 mg in 0.1 M Mes, pH 5.0) was added to the disc to allow the peptide to covalently link to the disc, and the disc was placed at RT for 30 min. The disc was then washed five times with water and five times with trifluoroacetic acid to remove unbound peptide. The disc was then washed three times with methanol, and subjected to Edman degradation. The disc was treated at 50 °C for 10 min with 0.5 ml of coupling reagent (methanol:water:triethylamine: phenylisothiocyanate; 7:1:1:1, v/v). After five washes with 1 ml of methanol, the disc was treated at 50 °C for 6 min with 0.5 ml trifluoroacetic acid to cleave the amino-terminal amino acid. The trifluoroacetic acid solution was placed in a scintillation vial and the disc was washed with 1 ml of trifluoroacetic acid and 42.5% phosphoric acid (9:1, v/v). The wash was combined with the trifluoroacetic acid solution, and the released ^{32}P was determined by Cerenkov counting. At this stage, the disc was either stored in methanol at −20 °C or washed five times with 1 ml of methanol before the next cycle was started.

Preparation and Isolation of Peptides for Sequencing—Purified baculovirus expressed PR-B (10 μg) was digested with trypsin (0.5 μg). Tryptic peptides were separated by reverse phase HPLC. Fractions with retention times corresponding to ^{32}P-labeled tryptic phosphopeptides from T47D cells were collected, dried in a Speedvac, and sequenced using an automated sequencer (23).

In Vitro Phosphorylation of PR-B with Casein Kinase II—Purified baculovirus expressed PR-B was incubated at 37 °C for 30 min in a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$,

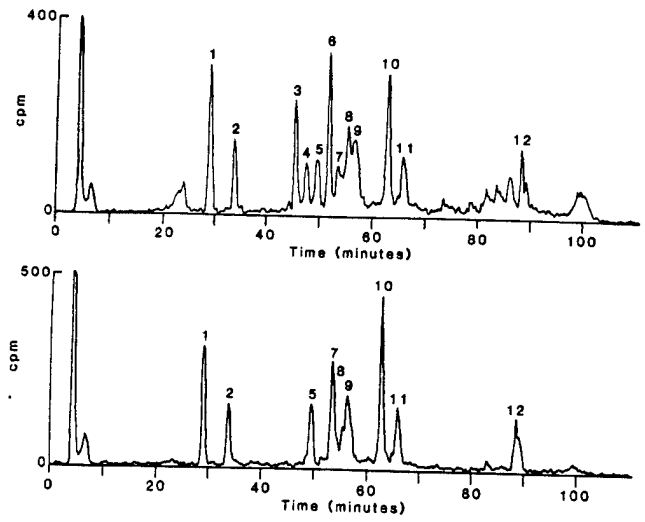

FIG. 1. **Phosphopeptide maps of PR isolated from hormone-treated T47D cells.** The tryptic phosphopeptides prepared from immunopurified PR-A and PR-B were separated by C18 reverse phase HPLC and detected with an on-line radioactivity detector. The major peaks have been designated *1–12*. Although not all of these peptides are detected in every experiment, these are the peptides most commonly seen. *Upper panel*, elution pattern of PR-B; *lower panel*, elution pattern of PR-A.

10 μM [γ-^{32}P]ATP (specific activity, 100,000 cpm/pmol), and 5 units of casein kinase II. ^{32}P-Labeled PR-B was separated by SDS-gel electrophoresis and detected by autoradiography. The band containing PR-B was excised and subjected to subsequent analyses consisting of tryptic digestion, HPLC and peptide gel separations, protease digestion, and manual ^{32}P release.

RESULTS

Tryptic Phosphopeptide Maps of Human PR—PR-A and PR-B were metabolically labeled to steady-state in T47D cells as described previously (11). Radiolabeled receptors were immunoprecipitated from cell lysates, and the A and B receptor isoforms were separated by SDS-gel electrophoresis and eluted from the gel pieces by digestion with trypsin. The eluted phosphopeptides were then separated by HPLC on a C18 reverse phase column. Shown in Fig. 1 are tryptic phosphopeptide maps of PR-A (*lower panel*) and PR-B (*upper panel*) isolated from cells that were treated for 2 h with the synthetic progestin R5020. The phosphopeptide maps therefore represent the fully phosphorylated state of hPR. Peaks with assigned numbers are the abundant phosphopeptides that have been observed most consistently. PR-B contains at least 12 tryptic phosphopeptides, and a minimum of nine phosphopeptides are common to PR-A and PR-B. The three peptides designated 3, 4, and 6 are unique to PR-B.

Alkaline Polyacrylamide Gel Electrophoresis of Tryptic Phosphopeptides—Tryptic phosphopeptides of PR-A and PR-B were separated by HPLC and collected in fractions. Those fractions corresponding to each of the major peaks were pooled, dried, and electrophoresed on alkaline 40% acrylamide gels (Fig. 2). While most of the peaks contain only one peptide, we found that several of the peaks (*4B*, *5A*, *7B*, *8,9A*, and *8,9B*) actually contain multiple peptides that were not resolved by reverse phase HPLC. These data suggest that there may be more phosphorylation sites than indicated by the HPLC analysis alone. While most peptides are common to PR-A and PR-B, several are unique to PR-B (*3B*, *4B*, and *6B*) and one (*5A*) may be unique to PR-A.

Identification of Phosphorylation Sites: Ser^{81}, Ser^{162}—As a first step to identify the major PR-B specific sites, tryptic peptide 3 and peptide 6, after isolation from HPLC, were redigested with two other endoproteinase, Asp-N and Glu-C. This was followed by alkaline peptide gel electrophoresis. Fig. 3 shows that the mobility of peptide 3 increased after either Asp-N or Glu-C digestion, indicating that peptide 3 contains both Asp and Glu. The mobility of peptide 6 remained the same after enzyme digestion, indicating the absence of Asp and Glu in peptide 6. These secondary digestions of tryptic phosphopeptides are useful for narrowing the possible sequence locations for peptide 3 and 6.

In addition, to determine the position of the phosphoamino acid in tryptic peptides 3 and 6, manual Edman degradation was performed. Since HPLC peaks 3 and 6 each contain only one phosphopeptide as judged by alkaline gel electrophoresis (Fig. 2), samples from the HPLC analysis were used for this experiment. Fig. 4 shows the cycle at which ^{32}P was released. The majority of the ^{32}P in peptide 3 and peptide 6 was released at cycle 8 and cycle 3, respectively.

All the tryptic peptides in PR-B that have a serine in cycle 8 or cycle 3 and thus have the potential to release ^{32}P in the manner observed are listed in Table I. There are three possible peptides that have serines in position 8 and that also have sites for cleavage by Asp-N and Glu-C. These are the peptides that start with residue 63, 74, and 547. Since this peptide is found only in PR-B, it is likely that it is either the peptide beginning with residue 63 or with residue 74. Peptide 6 contained a phosphoserine in cycle 3 and was not cut by Asp-N or by Glu-C. The peptide starting with residue 160 is the only peptide located in the unique NH_2 terminus of PR-B which contains a serine in the third position, that also lacks Asp-N and Glu-C digestion sites. Thus, we concluded that Ser^{162} is the only possibility for phosphorylation of peptide 6.

To distinguish between the two likely possibilities for peptide 3 and to confirm the deduced identity of peptide 6, we have performed amino acid sequencing of peptide 3 and 6 using purified baculovirus PR-B as the source of receptor. After trypsin digestion, peptides were separated by HPLC and fractions which had the same retention time as either [^{32}P]phosphopeptide 3 or peptide 6 of PR-B from T47D cells were collected, and the amino acid sequence was determined by an automated microsequencer. The sequencing results shown in Table II reveal that peptide 3 contains the sequence beginning with residue 74 identifying the phosphorylation site as Ser^{81}; peptide 6 contains the sequence beginning with residue 160. A few minor amino acid sequences, either from trypsin or receptor, were found in the peptide 3 and peptide 6 preparations. However, these sequences did not match with the ^{32}P release and endoproteinase digestion results. Thus, the secondary protease digestion, ^{32}P release, and direct amino acid sequencing results collectively provide unambiguous identification that Ser^{81} and Ser^{162} are phosphorylated *in vivo*.

In Vitro Phosphorylation of PR-B—We noticed that Ser^{81} is within a casein kinase II (CKII) phosphorylation motif; therefore, we decided to test CKII for its ability to phosphorylate PR-B *in vitro*. As a substrate for *in vitro* kinase assays we utilized a highly purified preparation of human PR-B expressed as a full-length recombinant protein in a baculovirus system (27). The receptor was phosphorylated as described under "Materials and Methods" and separated by SDS-gel electrophoresis. Phosphorylated PR-B was detected by autoradiography and quantified by counting the radioactivity in the band containing labeled PR-B. The stoichiometry of receptor phosphorylation (>25%) was calculated based on the total amount of PR-B used and moles of phosphate incorporated. Fig. 5 shows that PR-B was readily phosphorylated only in the presence of CKII, indicating that there is no endogenous kinase activity

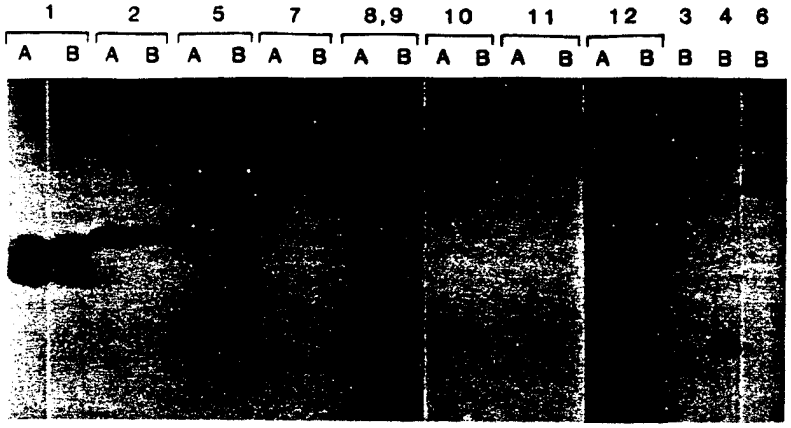

FIG. 2. **Alkaline polyacrylamide gel electrophoresis of tryptic phosphopeptides.** The phosphopeptides isolated by HPLC were separated by electrophoresis on a 40% polyacrylamide gel and detected by autoradiography. The PR-A (*A*) and PR-B (*B*) peptides with the same retention times on HPLC were run in parallel.

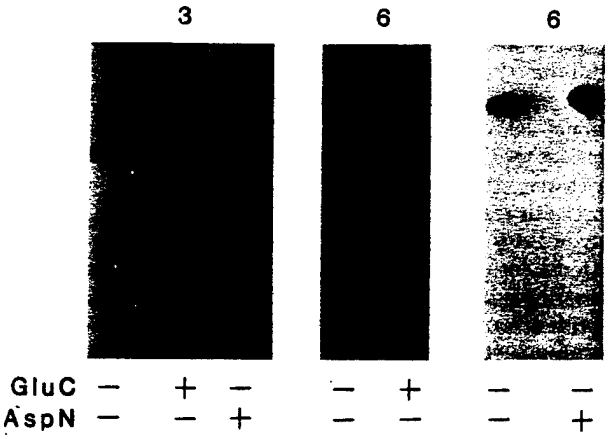

FIG. 3. **Characterization of tryptic phosphopeptides by redigestion with additional proteases.** The indicated tryptic peptides were treated with Asp-N which cleaves on the amino-terminal side of aspartic acid or with Glu-C which cleaves on the carboxyl-terminal side of glutamic acid and analyzed by polyacrylamide gel electrophoresis.

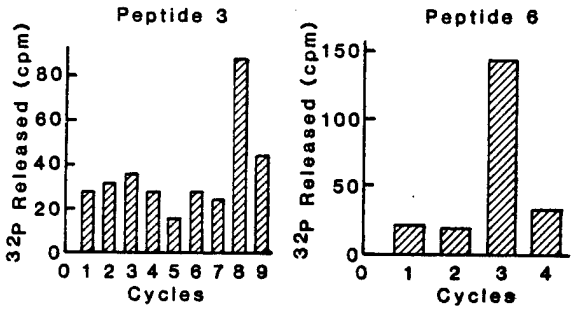

FIG. 4. **Identification of the cycle containing phosphoserine by manual Edman degradation.** Peptides 3 and 6 were covalently coupled to arylamine membrane discs using carbodiimide and subjected to manual Edman degradation. The radioactivity in the released amino acid was determined after each cycle using a scintillation counter. The background count (24 ± 4, $n > 10$) was not subtracted from all the counts. The cycle containing the released ^{32}P is the cycle containing the phosphoamino acid.

associated with purified PR-B, and that it is an excellent substrate for CKII. The tryptic phosphopeptide map of the *in vitro* phosphorylated PR-B shown in Fig. 6 reveals one major ^{32}P peak which corresponds to peptide 3 isolated from *in vivo* phosphorylated PR. The fraction was collected and dried, digested with Asp-N and Glu-C and run on a peptide gel. The mobility of this peptide treated or untreated with endoproteinase was the same as that of peptide 3 (data not shown). To further confirm the identity of this single major phosphopeptide, we performed manual Edman degradation. As shown in Fig. 7, ^{32}P was released in cycle 8 confirming that Ser^{81} was preferentially phosphorylated by CKII *in vitro*. In contrast, purified PR-A was not phosphorylated *in vitro* by CKII (data not shown), further confirming the preference of this enzyme for the PR-B specific Ser^{81}.

TABLE I

Potential tryptic phosphopeptide candidates for peptides 3 and 6

The number preceding the peptide indicates the position of the first amino acid of the peptide within the sequence of PR-B.

Phosphoserine in eighth position																
63	P	C	Q	G	Q	D	P	[S]	D	E	K					
74	T	Q	D	Q	Q	S	L	[S]	D	V	E	G	(A)	Y	S	R
95	G	A	G	G	S	S	S	[S]	P	P	E	K				
371	D	D	A	Y	P	L	Y	[S]	D	F	Q	P	P	(A)	L	K
547	P	D	S	E	A	S	Q	[S]	P	Q	Y	S	F	E	S	L	P	Q	K
Phosphoserine in third position																
160	V	L	[S]	P	L	M	S	R								
188	G	L	[S]	P	A	R										
271	E	D	[S]	R												
547	P	D	[S]	E	A	S	Q	S	P	Q	Y	S	F	E	S	L	P	Q	K
770	H	V	[S]	Q	M	L	Y	F	A	P	D	L	I	L	N	E	Q	R	
791	E	S	[S]	F	Y	S	L	C	L	T	M	W	Q	I	P	Q	E	F	V	K
900	A	L	[S]	V	E	F	P	E	M	M	S	E	V	I	A	A	Q	L	P	K

DISCUSSION

We have identified two PR-B-specific phosphorylation sites, Ser^{81} and Ser^{162} located within the 164-amino acid amino terminus of PR-B. We have also found that casein kinase II preferentially phosphorylates Ser^{81} *in vitro*. Analysis of phosphopeptide maps of PR from hormone-treated T47D cells was previously reported by Sheridan (8) who indicated that there might be at least five common phosphopeptides between PR-A and PR-B with a single site unique to PR-B in the absence of hormone and one or two more additional ones after hormone treatment. Our study has shown that hPR phosphorylation may be more complex than initially reported. We find at least 12 phosphopeptides including the B-specific peptides. The difference in the total number of peptides may be due to our use of an on-line radioactive flow detector, which gives higher resolution than counting individual fractions after HPLC.

Analysis of phosphorylation of steroid receptors has been hampered by both the complexity of the phosphorylation (30, 31) and the low abundance of receptor both of which make the use of conventional protein chemistry techniques to identify the sites both very expensive and difficult. Phosphorylation sites in chicken progesterone receptors, isolated from ^{32}P-labeled oviduct tissue minces (23) and in mouse glucocorticoid receptors (32) overexpressed in Chinese hamster ovary cells, have been identified by HPLC isolation of phosphopeptides followed by amino acid sequencing. Some of the phosphorylation sites in

TABLE II
Peptide sequence analysis
Peptide 3 and 6 were sequenced as described in the text.

Cycle	Amino acid	Position[a]	
			pmol
Peptide 3			
1	Thr	74	29.8
2	Gln	75	34.3
3	Glu	76	18.2
4	Gln	77	32.1
5	Gln	78	39.2
6	Ser	79	7.9
7	Leu	80	28.1
8	Ser	81	6.5
9	Glu	82	13.3
10	Val	83	28.0
Peptide 6			
1	Val	160	11.7
2	Leu	161	24.7
3	Ser	162	1.7
4	Pro	163	5.5
5	Leu	164	7.8
6	Met	165	2.5
7	Ser	166	1.4
8	Arg	167	1.1

[a] Amino acid position within the PR-B.

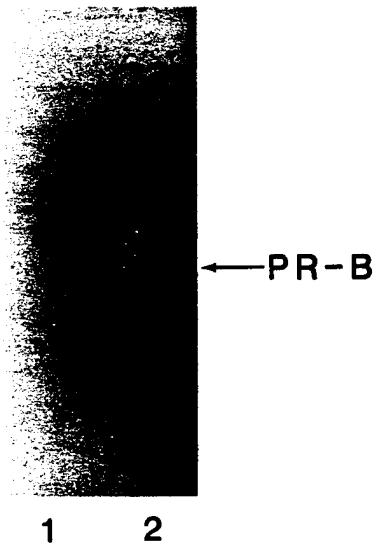

FIG. 5. ***In vitro* phosphorylation of PR-B by casein kinase II.** Phosphorylation of PR-B was done under the conditions described in the text of Methods. After incubating at 37 °C for 30 min, the reaction was terminated by addition of sample buffer, heated at 90 °C for 5 min, separated by SDS-gel electrophoresis, and autoradiographed. *Lane 1*, reaction without casein kinase II; *lane 2*, reaction with casein kinase II. Position of PR-B is indicated.

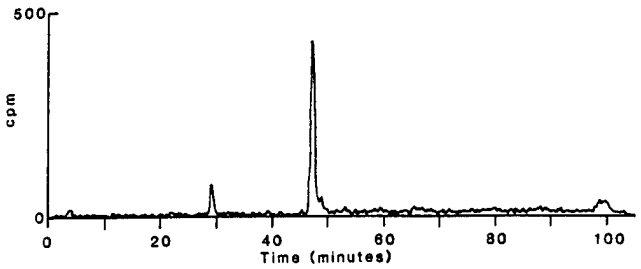

FIG. 6. **Phosphopeptide map of PR-B phosphorylated *in vitro* with CKII.** Phosphorylated PR-B was isolated by SDS-gel electrophoresis, digested with trypsin, and separated by reverse phase HPLC. The elution time of the major peak corresponds to that of peptide 3. The elution time differs somewhat from that in Fig. 1 but was confirmed by comparison with known phosphopeptides.

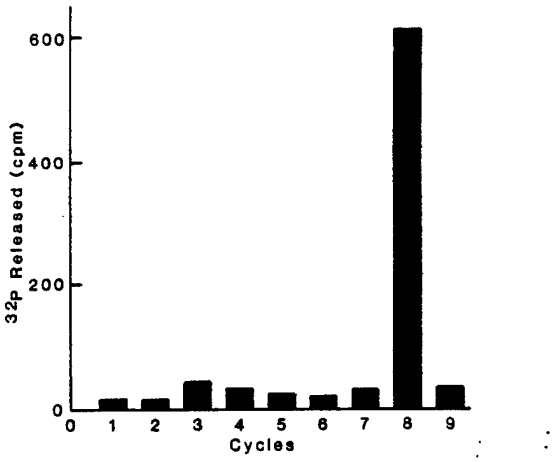

FIG. 7. **Manual ^{32}P release of peptide CKII.** Peptide CKII was subjected to nine cycles of manual ^{32}P release using the procedure described under "Materials and Methods."

the estrogen receptor have been identified by site directed mutagenesis. Regions of estrogen receptor were deleted and phosphorylation measured to locate regions containing phosphorylation sites. Potential candidate serine residues within those regions were mutated and confirmation that the substitutions actually result in a loss of expected phosphopeptide was done by phosphopeptide mapping. The limitation of this approach is that deletion of a region and/or mutation of an amino acid may alter the structure of the protein in a way that results in reduced phosphorylation at a distal site. As reported for the vitamin D receptor (33), mutation of an authentic site can in fact result in phosphorylation of an alternate site. The approach described in this paper permits direct, unambiguous identification of phosphorylation sites in wild type, endogenously expressed receptors. Use of secondary protease digestion of tryptic phosphopeptides isolated from HPLC as well as release of ^{32}P by manual Edman degradation, enabled us to identify one of the major phosphorylation sites located in the unique NH_2 terminus of PR-B (Ser^{162}) and narrowed the possibilities for the second (Ser^{81}) to two sites. Amino acid sequencing of corresponding tryptic peptides isolated from baculovirus expressed PR-B provided confirmation of Ser^{162} and identified Ser^{81} as the second site. However, if the carrier protein were unavailable we could have performed manual Edman degradation on the Asp-N digested peptide 3 to distinguish between the two remaining possibilities. Phosphopeptide mapping has shown that baculovirus expressed hPR is correctly phosphorylated on all sites but one (peptide 9)[2] and thus is a suitable carrier protein for identification of all other sites by amino acid sequencing of phosphopeptides, including peptides 3 and 6 in this study. Hilliard *et al.* (33) successfully used vitamin D receptor isolated from a yeast expression system as a carrier protein for amino acid sequencing to identify a phosphorylation site in the human vitamin D receptor, and chicken PR expressed in yeast was found to be correctly phosphorylated on all of the sites that have been identified in the endogenously expressed receptor (34). Thus, correct phosphorylation of steroid receptors expressed as a recombinant protein in heterologous eukaryotic systems, such as baculovirus or yeast, may be more the rule than the exception. Therefore, the approach developed in our

[2] C. A. Beck, Y. Zhang, N. L. Weigel, and D. P. Edwards, manuscript in preparation.

study of using endogenously expressed ^{32}P-labeled protein as a tracer combined with unlabeled carrier protein purified from an overexpression system for amino acid sequencing may be a generally effective means to identify phosphorylation sites on steroid receptors and other proteins.

The identification of the major phosphorylation sites located in the unique NH_2 terminus of hPR-B is a potentially important finding that may help to explain the distinct functional properties of the two hPR isoforms that appear to be dependent upon cell type-specific factors and on target promoter context (20–22). Of particular interest is the recent discovery that PR-A can under certain circumstances act as a repressor of transcription mediated by PR-B as well as other steroid receptors in the closely related glucocorticoid receptor subfamily (22). The mechanism of the repressor activity of PR-A is unknown. By performing site-directed mutagenesis of Ser^{81} and Ser^{162}, it will now be possible to determine to what extent phosphorylation of these NH_2-terminal sequences in PR-B contribute to the distinctly different biological activities of the two PR isoforms. Is it possible that PR-B lacking phosphorylation of Ser^{81} and Ser^{162} will behave in a manner more closely resembling that of PR-A than wild type PR-B? It is also worth noting how the phosphorylation states of cPR and hPR differ. Although cPR is expressed as an A and B isoform, no PR-B-specific phosphorylation sites have been detected. As shown here, hPR phosphorylation is much more complex exhibiting as many as 9–12 sites compared to 4 sites in cPR. Additionally, all hPR phosphorylation sites appear to be located in the A/B region NH_2-terminal to the DNA binding domain (8),[3] whereas a major hormone-dependent site that is involved in modulation of cPR function is located in the COOH terminus between the DNA and steroid binding domains.[4] This illustrates the importance of phosphorylation site identification for individual steroid receptors and that one may not be able to extrapolate phosphorylation site data between even closely related receptors such as cPR and hPR.

A computer search of hPR revealed that Ser^{81} is in one of 11 potential CKII sites (*X*-Ser/Thr-*X*-*X*-Glu/Asp) (35). CKII is a ubiquitous multifunctional enzyme which phosphorylates and regulates a variety of proteins including those involved in the regulation of transcription and translation (36). CKII may also play a role in regulating steroid receptors. It was reported that the thyroid hormone receptor encoded by the chicken c-*erbA*α gene is phosphorylated at a single site by CKII (37). In addition, a CKII site in human vitamin D receptor has been identified as a phosphorylation site both *in vivo* and *in vitro* (33, 38). We therefore tested the ability of CKII to phosphorylate PR *in vitro*, finding that CKII preferentially phosphorylated Ser^{81} without phosphorylating any of the other potential CKII sites in hPR.

Ser^{162} is one of 15 potential phosphorylation sites in hPR that fit the consensus motif (*X*-Ser/Thr-Pro-*X*) of the proline-directed kinases. The S/T-P motifs are largely located in the amino-terminal region of PR, the region considered important for interaction with other transcription factors. Interestingly, five Ser-Pro sites are located in the B specific amino terminus of PR. It is possible that these S/T-P motifs play a role in PR interaction with other transcriptional factors and B specific functions. It is intriguing to note that many of the identified phosphorylation sites in steroid receptors such as chicken PR, human estrogen receptor, and mouse glucocorticoid receptor also contain Ser-Pro motifs which are part of the consensus sequences for mitogen-activated protein (MAP) kinases and cyclin-dependent kinases. MAP kinases, present in both cytoplasm and nucleus, are important intermediates in signal transduction pathways that are initiated by many types of cell surface receptors. There is evidence that MAP kinases play a key role in the transduction of signals through both protein kinases and protein phosphatases. Whether MAP kinases regulate the activity of PR remains to be established. Ser-Pro is also a minimum consensus for cyclin-dependent kinases; cyclins and cyclin-dependent kinases are key regulators of cell cycle progression in eukaryotic cells. There is also evidence that progesterone receptor regulates expression of cyclin genes in T47D cells (39, 40). The data suggest that the activity of human PR may be regulated by different kinases that are actively involved in either signal transduction or cell cycle regulation and that, in turn, PR may regulate proteins involved in cell cycle control.

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Identification of a Group of Ser-Pro Motif Hormone-Inducible Phosphorylation Sites in the Human Progesterone Receptor

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The human progesterone receptor (PR) is a member of the steroid/thyroid hormone superfamily of nuclear receptors. The receptor is expressed as two forms, PR-B and the shorter PR-A, which lacks the NH2-terminal 164 amino acids of PR-B; whereas PR-B seems to be predominantly a transcriptional activator, PR-A also functions as a repressor. Our previous studies of PR expressed in T47D breast cancer cells have shown that PR is a phosphoprotein whose phosphorylation is enhanced in response to hormone. There is an initial rapid (minutes) increase in phosphorylation followed by a slower, less substantial increase, which results in decreased mobility of the receptor on sodium dodecyl sulfate gels. We now report the identification of three phosphorylation sites, which are predominantly phosphorylated during the later phase of the response to hormone. These sites, Ser^{102}, Ser^{294}, and Ser^{345}, are all found in Ser-Pro consensus sequences. Whereas Ser^{294} and Ser^{345} are common to PR-A and PR-B, Ser^{102} is unique to PR-B. Finally, we demonstrate that phosphorylation of Ser^{345} is associated with the altered mobility on sodium dodecyl sulfate gels. (Molecular Endocrinology 9: 1029–1040, 1995)

INTRODUCTION

The progesterone receptor (PR) is a member of the superfamily of ligand-inducible transcriptional activators. PR from several species is expressed as two distinct molecular forms, PR-A and PR-B, which have been shown to have different functional capabilities dependent on the cellular and target gene context (1–3). The B form of the receptor has an extra N-terminal amino acid sequence [128 amino acids for chicken PR (cPR) and 164 amino acids for human PR (hPR)]; otherwise, A and B receptors are identical throughout the remainder of the molecule, including the centrally located DNA-binding domain and the C-terminal ligand-binding domain (LBD). The N-terminal domain of PR contains sequences responsible for transcriptional activity, and a second hormone-dependent transcriptional activation function resides in the LBD. All steroid receptors that have been examined are phosphoproteins, and most show an increase in overall phosphorylation in response to binding hormone in the intact cell (*in vivo*) (4). Most steroid hormone receptors have also been shown to be phosphorylated on multiple serine residues in the N-terminal domain (5–17). Exceptions are reports of a phosphotyrosine in the human estrogen receptor (hER) (18) and tyrosine phosphorylation of retinoic acid receptor-β (19). Additionally, single phosphoserine residues have been detected outside of the N-terminus, including sites in the hinge region (5) (between the DNA-binding domain and the LBD) of chicken oviduct PR and vitamin D receptor (VDR) (20).

The functional role for steroid receptor phosphorylation remains poorly defined. The fact that most sites are located in the N-terminal domain suggests a role in regulating transcriptional activity; however, phosphorylation could be involved in other receptor functions such as DNA and steroid binding or in modulating interaction with other cellular proteins (21). In support of a role in modulating transcriptional activity, mutation of single phosphorylation site(s) in the hER to nonphosphorylatable amino acids reduced ER-mediated gene transcription in transfection studies (13, 14). There is also some reduction in transcriptional activity of glucocorticoid receptors mutated at multiple phosphorylation sites (22). Studies with phosphorylation

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site mutants in cPR revealed the importance of the hinge site (Ser^{530}) for receptor sensitivity to steroid induction of transcriptional activity (21). Additional evidence that phosphorylation has a role in function has been provided by a number of studies showing that various cellular modulators of other signal transduction pathways can activate the cPR, as well as other steroid receptors, in the absence of steroidal ligand (23–27). These include agents such as epidermal growth factor, protein phosphatase inhibitors (okadaic acid, calyculin, and vanadate), and compounds that elevate intracellular cAMP (28) as well as the neurotransmitter dopamine (24). Whether ligand-independent activation by these alternate signaling pathways is mediated directly by effects on the phosphorylation state of the steroid receptor or indirectly through phosphorylation of other cellular factors remains largely unresolved. Available data suggest that the mechanism for ligand-independent activation may be dependent on the receptor type and cellular context (25, 28–31). Curiously, glucocorticoid receptors (GRs) and hPR seem to be much less susceptible to ligand-independent activation than other classes of steroid receptors. Thus far, there is one report of activation of hPR or GR in COS-1 cells by 8-bromo-cAMP in the absence of ligand that seemed to be promoter specific (32). In contrast, several other studies have failed to detect ligand-independent activation of hPR but have reported potentiation of hormone-dependent activation by various modulators of protein phosphorylation (29, 31), as well as functional switching of RU486 from a potent PR antagonist to a partial agonist by cotreatment of cells with 8-bromo-cAMP (33, 34).

Phosphorylation of hPR is complex. Earlier studies by Sheridan *et al.* (35) showed by HPLC analysis that at least five phosphotryptic peptides were generated from hPR form A, and a sixth phosphopeptide was detected in PR form B. Using higher resolution techniques, we have detected at least six phosphotryptic peptides from PR-A and nine in PR-B (36). This suggests that three phosphorylation sites are located within the unique N-terminal segment of PR-B, and we have identified two of these as Ser^{81} and Ser^{162} (36). Consistent with the multiplicity of phosphorylation sites, hormone-induced hyperphosphorylation of hPR seems to be an ordered process. Analysis of the time course of progestin effects has revealed that phosphorylation takes place in at least three distinct steps: basal phosphorylation in the absence of hormone, a rapid net increase in phosphorylation that occurs within a few minutes of progestin addition, followed by phosphorylation that promotes a decrease in electrophoretic mobility (or upshift) of PR on sodium dodecyl sulfate (SDS) gels (29). Interestingly, the upshift requires 40–60 min for completion and occurs with a small additional increase in net phosphorylation, suggesting this is due to changes in a single (or only a few) phosphorylation sites (29). Studies by Takimoto *et al.* (37), with a zinc finger DNA-binding mutant and with the progestin antagonist ZK98299, which impairs hPR DNA binding, suggested that this late-stage phosphorylation associated with the PR upshift may be both hormone and DNA-binding dependent.

In the present study we have used methods developed previously for the identification of the two major hPR-B-specific phosphorylation sites (36) to determine the effects of hormone on individual phosphorylation sites, questioning whether hormone stimulates phosphorylation of any major new sites and/or promotes a general increase of basal sites. We show that hormone has the dual effect of increasing phosphorylation of basal sites and inducing three new sites that are barely detectable in the absence of hormone. The hormone-inducible sites were identified as Ser^{102}, Ser^{294}, and Ser^{345}. All of these sites are within Ser-Pro motifs, and Ser^{102} is present only in PR-B. Additionally, these sites as a group were observed to be distinct from basal sites by their slower kinetics of increased phosphorylation and by the fact that the slowly occurring upshift of PR on SDS gels was associated only with phosphorylation of Ser^{345}.

RESULTS

Hormone Effects on Site-Specific Phosphorylation of hPR

PRs were metabolically labeled with [^{32}P]orthophosphate to steady state in T47D breast cancer cells and then were treated with the synthetic progestin R5020 for various times from 0–2 h. Whole-cell extracts were prepared in the presence of phosphatase inhibitors, and ^{32}P-labeled receptors were immunoprecipitated and separated by SDS polyacrylamide gel electrophoresis (PAGE). A and B receptors were separately eluted from gel pieces by digestion with trypsin, and the resultant peptides were isolated by HPLC on a C18 reverse-phase column. An on-line radiodetector was used for continuous monitoring of eluted ^{32}P-labeled peptides. Figure 1 shows the tryptic phosphopeptides of PR-A and PR-B obtained either in the absence of hormone addition (time zero) or after treatment of cells with R5020 for a short (5-min) or prolonged (2-h) time. The HPLC peaks that were assigned numbers are the major ^{32}P-labeled tryptic peptides observed consistently in repeated experiments. In its maximally phosphorylated state (2 h of R5020 treatment), PR-B contains numerous tryptic phosphopeptides, designated peaks 0–12. Four of these are unique to full-length PR-B (peaks 0, 3, 4, and 6), whereas the remainder (peaks 1, 2, 5, and 7–12) are common to PR-A and PR-B (Fig. 1).

As will be described later in more detail, not all the HPLC ^{32}P-labeled peaks represent distinct phosphorylation sites. Some minor peaks are a result of prolonged tryptic digestion, and some are a result of incomplete digestion. Thus, the number of sites cannot be determined simply from the HPLC profile. Based on the data presented below, the peaks

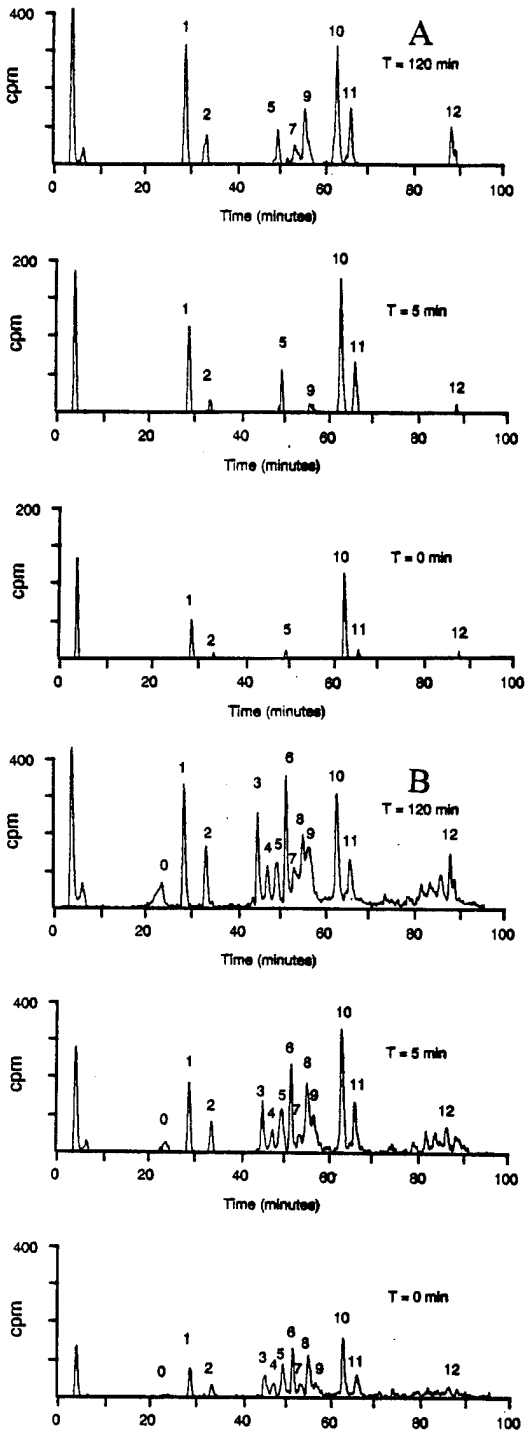

Fig. 1. Phosphopeptides of PR Isolated from Hormone-Treated T47D Cells

The tryptic phosphopeptides prepared from immunopurified PR-A and PR-B were separated by C18 reverse-phase HPLC and detected with an on-line radioactivity detector. The major peaks have been designated 1–12. A and B, Maps for tryptic phosphopeptides of PR-A and PR-B, respectively. Times 0, 5, and 120 min are the lengths of the R5020 treatment of T47D cells. The *top* profile of B is reproduced from Zhang *et al.* (36) (Fig. 1), with the permission of The American Society for Biochemistry & Molecular Biology, Inc.

designated as 7–9 represent heterogenous HPLC elution of what is apparently a single phosphopeptide, as does the series of peaks at the longest retention time (>80 min), which have collectively been designated as peptide 12 (Fig. 1). For convenience, peptides 7–9 are referred to in the subsequent text as peptide 9 only. The reason for the elution heterogeneity of these two phosphopeptides is not entirely clear but is likely due to oxidation products and to incomplete proteolysis. It should also be noted that the heterogeneity in peptides 9 and 12 is more pronounced in PR-B than in PR-A (Fig. 1).

Comparison of the HPLC mapping profiles of both PR-A and PR-B in the presence and absence of hormone showed that the majority of phosphopeptides are detected as basal sites in the absence of hormone, and that hormone treatment stimulated a rapid (within 5 min) general increase in ^{32}P labeling that was proportionately similar for each. These basal hormone-stimulated sites in PR-A are represented by peptides 1, 2, 5, 10, and 11 and in PR-B by peptides 1–6, 10, and 11. In contrast, phosphopeptides 9 and 12 in PR-A and 0, 9, and 12 in PR-B are barely detectable in the absence of hormone and exhibit a proportionately greater increase on hormone treatment than the basal sites. Thus, sites present in peptides 0, 9, and 12 seem to be largely hormone inducible. The kinetics of phosphorylation of these sites also seem to be different from that of basal hormone-stimulated sites. Levels of phosphorylation of peptides 0, 9, and 12 increase only slightly after a few minutes of hormone treatment; full phosphorylation requires 2 h of exposure to hormone (Fig. 1). Most of the basal sites reach their fully phosphorylated state within minutes of hormone addition (Fig. 1).

To obtain quantitative values for the effect of hormone on site-specific phosphorylation, we have determined the percent of total ^{32}P contained in each of the HPLC eluted peptides from PR-A and PR-B, taking the sum of the ^{32}P counts in all the major peaks as the total. Peak 2 was omitted from these analyses, because we have found that this peak contains an incomplete digestion product. Complete digestion results in a peptide that does not bind to the column and cannot be quantified. These calculations were made from repeated phosphopeptide mapping studies done under the same conditions as in Fig. 1. Analysis of the total incorporation of ^{32}P into PR-A and PR-B at 5 and 120 min of R5020 treatment shows that the majority of the net phosphorylation stimulated by R5020 occurs by 5 min of treatment (1.74- to 1.91-fold) and is completed (2.72- to 2.98-fold) by 120 min. Table 1 shows the relative changes in phosphorylation of the individual peptides in which a value of 1 indicates no relative increase in phosphorylation. Boxed numbers are the values for the sites that show substantial relative increases in phosphorylation. At 2 h of treatment, there is little relative change in phosphorylation of phosphopeptides except for peptides 0, 9, and 12. The value for most peptides was 1.0 or less, whereas

Table 1. Relative ^{32}P Labeling of Tryptic Phosphopeptides

HPLC peaks	Relative fold-increase ^{32}P labeling
PR-A P1	0.93
(n = 8) P5	0.71
P9	1.86
P10	0.63
P11	0.80
P12	1.98
PR-B P0	1.30
(n = 9) P1	1.18
P3	1.05
P5	0.84
P6	1.07
P9	1.47
P10	1.08
P11	0.74
P12	1.55

The relative fold-increase in phosphorylation of each phosphopeptide was calculated as the fold-change in percent of ^{32}P contained in each peptide using the sum of the counts in the major HPLC peaks indicated as the total. The values are the relative fold-change after 2 h of R5020 treatment. Values are averages from multiple experiments (n = 8 for PR-A, and n = 9 for PR-B). Because peptide 0 is a minor peak, we did not obtain quantitive data from earlier experiments, thus for this site, n = 4. Boxed numbers are those that exhibited substantial increases in relative phosphorylation.

peptides 0, 9, and 12 consistently gave values of more than 1.0. The only possible exception to this is peptide 1 that gave a relative increase of 1.18, but only in PR-B. In PR-A, the relative fold-increase of peptide 1 was less than 1.0. These quantitative analyses support the conclusion that basal sites exhibit a general and proportionately similar increase in response to hormone, whereas peptides 0, 9, and 12 respond differently, exhibiting greater increase relative to other sites. It is of interest to note that the differential hormone stimulation of peptides 9 and 12 was more pronounced for PR-A than PR-B, giving relative increases of 1.86 and 1.98 for peptides 9 and 12, respectively, in PR-A and values of 1.47 and 1.55, respectively, in PR-B (Table 1). In part, this is due to the smaller number of sites in PR-A. Peptide 0 is a minor peak unique to PR-B and was more difficult to quantitate. Nonetheless, it appears also to be distinct from basal sites. These results taken together suggest that peptides 0, 9, and 12 represent a class of phosphorylation sites in hPR that are distinct from the others. We therefore sought in the present study to identify the specific phosphorylation sites contained in these peptides.

Identification of Hormone-Dependent Phosphorylation Sites

To identify the amino acid residues that are phosphorylated in peptides 0, 9, and 12, methods were used that we had developed previously to identify two of the major PR-B-specific phosphorylation sites (36). This involved the combined approaches of manual Edman degradation of HPLC-isolated phosphopeptides to identify the position of the phosphoamino acid within a peptide, secondary proteinase digestions with Glu-C and Asp-N to identify the presence or absence of a Glu or Asp residue in a cleavable sequence, and conventional amino acid sequencing using purified *Baculovirus*-produced recombinant PR as a carrier (36). Manual Edman degradation of peptides 0, 9, and 12 was performed, and Fig. 2 shows the cycles at which ^{32}P was released. In each case, the majority of ^{32}P (>85%) was released during a single cycle, indicating that each peptide contains a single phosphorylation site. Release of ^{32}P was obtained at cycles 8, 2, and 1, respectively, for peptides 0, 9, and 12. Because previous studies (38) have indicated that hPR is phosphorylated exclusively on serine residues, we aligned all of the possible PR-B tryptic peptides that have a serine in either position 8, 2, or 1 as candidates for the three tryptic phosphopeptides 0, 9, and 12. As shown in Table 2, there are five possible tryptic peptides with

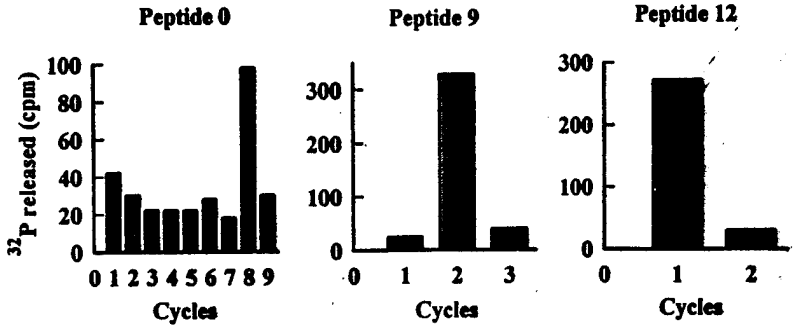

Fig. 2. Identification of the Position of the Phosphoamino Acids by Manual Edman Degradation

Peptides 0, 9, and 12 were covalently coupled to arylamine membrane discs using carbodiimide and subjected to manual Edman degradation. The radioactivity in the released amino acid was determined after each cycle using a scintillation counter. The background counts (24 ± 4, n >10) were not subtracted from the counts. The cycle containing the released ^{32}P is the cycle containing the phosphoamino acid.

Table 2. Tryptic Peptides for Phosphopeptides 0, 9, and 12

Phosphoserine in eighth position (candidates for peptide 0)	
63	P C Q G Q D P **S** D E K
74	T Q D Q Q S L **S** D V E G A Y S R
95	G A G G S S S **S** P P E K[a]
371	D D A Y P L Y **S** D F Q P P A L K
547	P D S E A S Q **S** P Q Y S F E S L P Q K
Phosphoserine in second position (candidates for peptide 9)	
107	D **S** G L L D S V L D T L L A P S G P G Q S Q P S P P A
	C E V T S S W C L F G P E L P E D P P A A P A T Q R
212	P **S** P Q A A A V E V E E E D G S E S E E S A G P L L K
275	F **S** A P R
344	S **S** P C A S S T P V A V G D F P D C A Y P P D A E P K[a]
732	W **S** K
791	E **S** S F Y S L C L T M W Q I P Q E F V K
846	S **S** Y I R
Phosphoserine in first position (candidates for peptide 12)	
168	**S** G C K
294	**S** P L A T T V M D F I H V P I L P L N H A L L A A R[a]
344	**S** S P C A S S T P V A V G D F P D C A Y P P D A E P K
400	**S** P R
403	**S** Y L V A G A N P A A F P D F P L G P P P P L P P R
735	**S** L P G F R
767	**S** Y K
837	**S** Q T Q F E E M R
846	**S** S Y I R

The number preceding the peptide indicates the position of the first amino acid of the peptide within the sequence of PR-B.
[a] Correct peptides.

a serine at position 8, seven with a serine at position 2, and nine with a serine as the first residue.

To identify which of the five candidates listed in Table 2 corresponds to phosphopeptide 0, we subjected the ^{32}P-labeled peptide (from 2-h hormone-treated T47D cells) to secondary digestion with two other endoproteinases, Asp-N and Glu-C, which cut, respectively, at the N-terminal side of Asp residues and the C-terminal side of Glu residues. The digested peptide was then submitted to gel electrophoresis on alkaline 40% polyacrylamide gels that are capable of resolving small peptides (39), to determine whether it contains a cleavable Asp or Glu bond based on a change in electrophoretic mobility. As shown in Fig. 3, the mobility of peptide 0 was unchanged after digestion with either Asp-N or Glu-C, indicating the lack of either an Asp or Glu residue. This secondary digestion therefore eliminates the peptides (Table 2) beginning with amino acids 63, 74, 371, and 547. They all have multiple Glu (single letter code E) and/or Asp (single letter code D) residues, which would produce altered-mobility [^{32}P]peptides on secondary digestion with Glu-C or Asp-N. Moreover, peptide 0 is PR-B specific, which provides a second criterion to eliminate the peptides beginning with amino acids 371 and 547 (Table 2). Finally, the peptide beginning with amino acid 74 has already been identified as corresponding to HPLC tryptic peptide 3 that contains Ser^{81} as the phosphorylation site (36). This leaves only the peptide beginning with amino acid 95. Although this peptide contains a single Glu, it is adjacent to the carboxyl-terminal lysine, and as reported previously (40), this

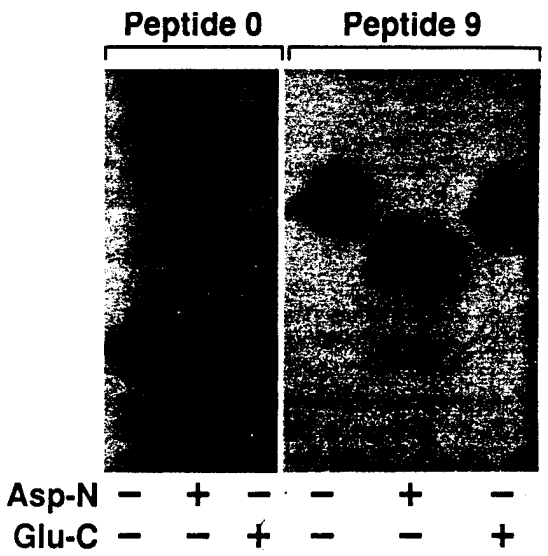

Fig. 3. Characterization of Endoproteinase-Treated Tryptic Phosphopeptides by Peptide Gel Electrophoresis

The ^{32}P-tryptic peptides as indicated in Fig. 1 were treated with Asp-N or Glu-C and analyzed by 40% alkaline polyacrylamide gel electrophoresis as described in *Materials and Methods*. The gel was dried, and peptides were detected by autoradiography.

We therefore conclude that tryptic phosphopeptide 0 corresponds to the peptide that begins with residue 95, which places the phosphorylation site at Ser^{102} (Table 2).

There are seven peptides that contain a Ser residue at position 2 (Table 2) and therefore are potentially peptide 9. Secondary digestion of peptide 9 with Asp-N increased its mobility on alkaline peptide gels, whereas Glu-C had no effect (Fig. 3). Moreover, Asp-N produced multiple bands suggesting the presence of more than one Asp residue in peptide 9. Similar results were obtained when we reran the secondary Asp-N-digested peptide 9 through reverse-phase HPLC. Peptide 9, which had a retention time of 56 min, was eluted earlier and seemed heterogeneous after Asp-N digestion but remained unchanged after Glu-C digestion, as shown in Fig. 4. These analyses narrow the peptides to the one starting with amino acid 344. All the other candidate peptides (Table 2) either lack Glu and Asp residues and should not be cut by either enzyme (peptides beginning with amino acids 275, 732, and 846), have Glu but not Asp residues (amino acid 791), or have multiple Glu and Asp residues and should be cut by either enzyme. It should be pointed out that although there is a Glu in the peptide starting with amino acid 344 that is two residues away from the C-terminus, it is next to a Pro. It has been reported that Glu-Pro bonds are not cleaved by Glu-C (40). Thus, we concluded that tryptic peptide 9 can be only the one starting with amino acid 344, placing the phosphorylated residue in cycle 2 as Ser^{345}.

There are nine candidates for tryptic peptide 12 that have a serine in position 1. Peptide 12 elutes near the end of the gradient in the reverse-phase HPLC column. As it turned out, this peptide is highly hydrophobic, which explained why we were unable to successfully isolate it for further analysis by secondary protease digestion and alkaline gel electrophoresis. Therefore, we used direct amino acid sequencing to identify this site, because very few peptides bind so tightly to the column, and, thus, a clean sequence can be obtained. This required the use of corresponding peptides from purified *Baculovirus*-expressed receptor. This approach has been successful in identifying Ser^{81} and Ser^{162} (36). In brief, *Baculovirus*-produced PR-B was purified to apparent homogeneity by immunoaffinity chromatography and then digested with trypsin (5% trypsin to receptor, wt/wt). The resulting tryptic peptides were separated by HPLC, and fractions that had the same retention time as [^{32}P]phosphopeptide 12 from T47D PR-B were collected. Amino acid sequencing of the eluted peptide was determined by an automated microsequencer. Results shown in Table 3 revealed two sequences. The major sequence beginning with amino acid 294 contains a Ser in the first position. Because the majority of the ^{32}P was released at cycle 1, as shown in Fig. 2, it is evident that Ser^{294} is phosphorylated. The minor sequence beginning with amino acid 280 resulted from incomplete digestion, yielding some dipeptide extending from Val^{280} to Lys^{370}. This is consistent with our finding that in one experiment out of three, the majority of ^{32}P was released at cycle 15 (as would be predicted for the dipeptide) instead of cycle 1. In the other two experiments, the majority of ^{32}P was at cycle 1. The resistance of peptides containing Lys or Arg followed by a negatively charged amino acid such as phosphoserine to cleavage with trypsin is well documented (40). Thus, the detection of both a completely and partially digested peptide is also consistent with the identifica-

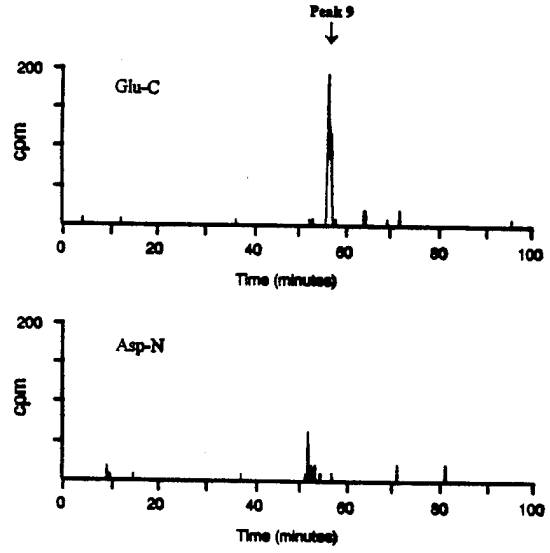

Fig. 4. Analysis of Endoproteinase-Treated Peptide 9 by Reverse-Phase HPLC

Peptide 9 was treated with either Asp-N or Glu-C and run on a C18 reverse-phase HPLC as described in Fig. 1. *Top*, Glu-C-treated peptide 9. *Bottom*, Asp-N-treated peptide 9. The *arrow* indicates the elution position of undigested peptide 9.

Table 3. Peptide Sequence Analysis

Cycle	Amino acid	Position	pmol
Major sequence			
1	Ser	294	2.41
2	Pro	295	2.36
3	Leu	296	2.23
4	Ala	297	2.55
5	Thr	298	0.26
6	Thr	299	0.17
7	Val	300	1.10
8	Met	301	0.37
9	Asp	302	1.27
10	Phe	303	0.58
Minor sequence			
1	Val	280	2.03
2	Ala	281	2.35
3	Leu	282	2.23
4	Val	283	1.15
5	Glu	284	0.69
6	Gln	285	0.53
7	Asp	286	0.93
8	Ala	287	0.57
9	Pro	288	0.90
10	Met	289	0.38

Peptide 12 was sequenced as described in the text.

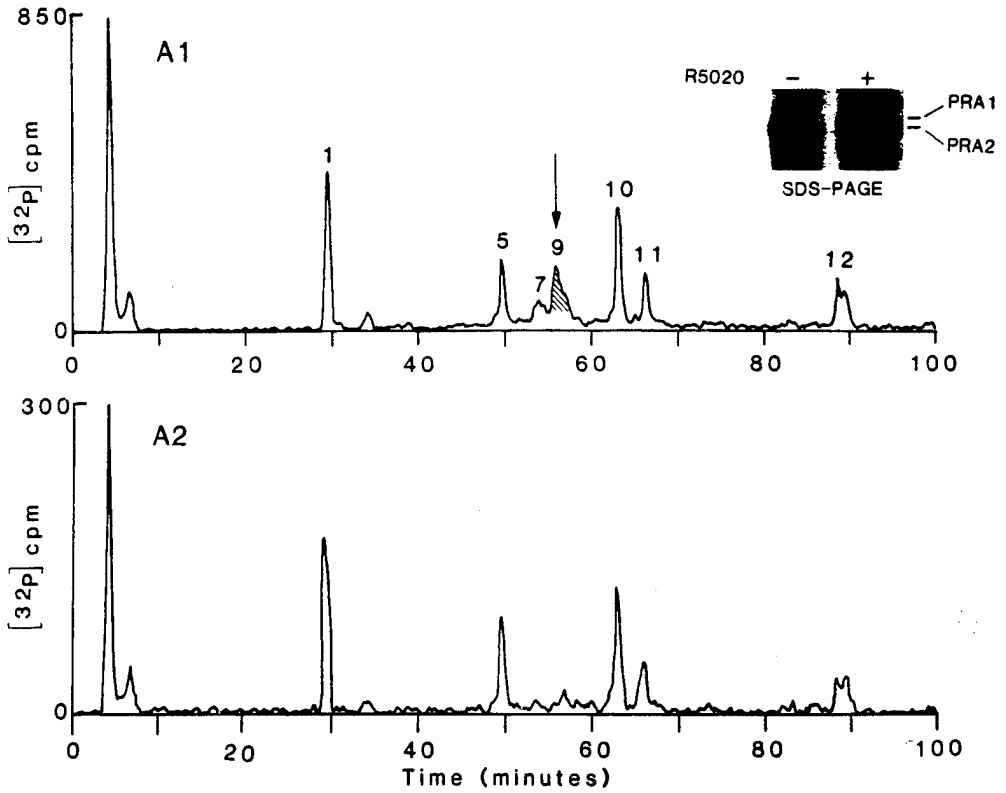

Fig. 5. Identification of Ser^{345} as the Phosphorylation Site Associated with Altered Mobility of PR

Receptors in T47D cells (2 × T75 flasks) were labeled to steady state with ^{32}P and then were treated with or without R5020 (40 nM) for 1 h at 37 C. Whole-cell extracts were prepared, and [^{32}P]PR was immunoprecipitated with AB-52 using Protein A Sepharose as the absorbent. In parallel, unlabeled PR from a larger number of cells (20 × T-175 flasks) were treated in the same manner. ^{32}P-Labeled PR and unlabeled PR were eluted from Protein A Sepharose and combined, and proteins were separated by SDS-PAGE. PR-A was detected by autoradiography of the wet gel (not shown) and by silver staining (*inset*). The faster- and slower-mobility PR-A bands are designated, respectively, A2 and A1. A1 and A2 from R5020-treated cells were excised and eluted from gels by digesting with trypsin, and the tryptic phosphopeptides were separated by HPLC on a C18 reverse-phase column. *Upper panel*, HPLC mapping of PR-A1; *lower panel*, PR-A2.

tion of Ser^{294} as the phosphorylation site in HPLC peak 12.

Identification of Ser^{345} as the Phosphorylation Site Associated with Altered Mobility of PR on SDS-PAGE

Human PR in T47D cells was labeled to steady state with ^{32}P, and cells were left untreated, or hormone (R5020) was added for 1 h to produce a partial upshift in the mobility of PR as detected by SDS-PAGE. The ^{32}P-labeled receptor was immunoprecipitated with AB-52, and in parallel, unlabeled PR from a larger number of T47D cells was treated with or without R5020 under the same conditions and was also immunoprecipitated with AB-52. The unlabeled PR was used as a carrier protein for detection of the upshifted and nonupshifted PR bands by silver staining. The immunoprecipitated ^{32}P-labeled and unlabeled PR were combined and submitted to SDS-PAGE. The wet gel was autoradiographed to confirm the position of the [^{32}P]A and B receptor bands and was then fixed and silver stained (29). Shown in the *inset* of Fig. 5 is the silver-stained SDS gel of PR-A from untreated and R5020-treated cells.

PR-A appeared as a single silver-stained band of approximately 94 kilodaltons in the absence of hormone but showed a second slightly slower mobility band after hormone treatment. Keeping in mind that the unlabeled PR was spiked with [^{32}P]PR, the slower (PR-A1), and faster (PR-A2) mobility bands of PR-A were excised, eluted from the SDS gel, and digested with trypsin, and the ^{32}P-labeled peptides were then separated by HPLC. As shown in Fig. 5, both PRA1 and PRA2 contained the same phosphopeptides in similar ratios except for peptide 9, which was present only in the slower mobility band (PR-A1). It should be noted that the other hormone-inducible phosphopeptide (number 12) that is common to PR-A and PR-B appeared in an equal amount in both the upper and lower bands, indicating that phosphorylation of Ser^{294} may not be required for the upshift. These results show that phosphorylation of Ser^{345} is associated with the upshift in

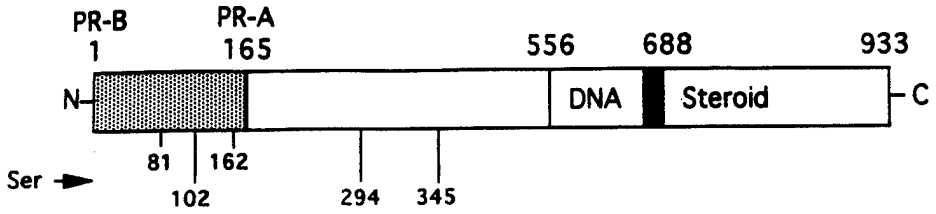

Fig. 6. Summary of hPR Phosphorylation Sites

Hormone-inducible sites indicated by the *extended arrow* are Ser^{102}, Ser^{294}, and Ser^{345}. Two of the hormone-inducible sites (Ser^{294} and Ser^{345}) are common to the A and B forms of PR, whereas Ser^{102} is unique to PR-B. Ser^{81} and Ser^{162} are previously identified sites that reside in the unique N-terminal segment of PR-B, and both are characterized as basal hormone-stimulated sites.

PR mobility on SDS gels, suggesting that this site alone may be responsible for the PR upshift.

DISCUSSION

An essential step for defining the functional role for steroid receptor phosphorylation and the protein kinases involved in postranslational modification of receptors is to identify the exact residues that are phosphorylated under different conditions of hormonal treatment. Knowledge of phosphorylation sites permits functional analysis by use of site-directed mutagenesis to change phosphoamino acids to nonphosphorylatable residues. We have taken the approach of identifying authentic phosphorylation sites in the wild type full-length receptor from cells (T47D) that express hPR endogenously as opposed to the use of deletion and truncation mutant receptors expressed in heterologous cells by transfection. The use of a wild type receptor avoids the potential problem of missing authentic sites or of detecting an inappropriate site due to potential conformational alterations in mutant receptors. As an example of the potential limitation of the mutagenesis approach to identify sites, Hilliard *et al.* (20) found that mutation of an authentic site in the full-length wild type VDR resulted in phosphorylation of an adjacent serine in the same phosphopeptide.

Using direct chemical analysis of hPR phosphopeptides isolated from ^{32}P-labeled T47D cells, we previously identified Ser^{81} and Ser^{162} as two major phosphorylation sites present in the unique N-terminal segment of PR-B (36). One of the sites (Ser^{81}) is a casein kinase II (CKII) consensus motif and is efficiently and specifically phosphorylated by CKII *in vitro*. The other PR-B-specific site (Ser^{162}) is contained in a Ser/Thr-Pro motif (36). Because hPR is phosphorylated basally, and hormone increases overall phosphorylation by 2-to 4-fold (29), we questioned in the present study whether hormone stimulates phosphorylation of any major new sites or simply increases phosphorylation of basal sites. Phosphopeptide mapping showed that hormone did not elicit the same relative increase in all phosphopeptides. Three peptides 0, 9, and 12, were barely detectable in the absence of hormone and exhibited a greater fold-increase on hormone treatment than other phosphopeptides. These became major phosphopeptides (*i.e.* a significant percentage of total ^{32}P labeling of PR) only after hormone addition. The hormone dependency of peptides 9 and 12 was particularly apparent in PR-A. In contrast, all other phosphopeptides (with the possible exception of peptide 1 in PR-B) were readily detectable as major sites in the absence of hormone, and the effect of hormone was to stimulate a general increase that was proportionately similar for this group of phosphopeptides. Thus, we conclude that the hPR contains at least two groups of phosphorylation sites based on response to hormone: basal sites that exhibit a general increase in phosphorylation, and hormone-dependent sites that largely require induction. These three sites are also distinguished as a group by the kinetics of response to hormone. Hormonal stimulation of basal sites is rapid, occurring within a few minutes, whereas phosphorylation of the inducible sites is slow, taking as long as 1–2 h for completion. Additionally, our phosphopeptide mapping of upper and lower mobility PR-A bands indicates that phosphorylation of Ser^{345} is uniquely associated with the upshift of PR on SDS gels. This hormone-inducible characteristic also sets Ser^{345} apart from basal phosphorylation sites.

We identified the phosphorylated residues in the inducible peptides 0, 9, and 12, respectively, as Ser^{102}, Ser^{345}, and Ser^{294}. Thus, one of the inducible sites (Ser^{102}) is PR-B specific, and the other two (Ser^{294} and Ser^{345}) are present in both A and B. We were able to identify sites in phosphopeptides 0 and 9 by manual Edman degradation of ^{32}P-tryptic phosphopeptides of T47D PR, followed by secondary digestion with Glu-C and Asp-N to narrow the range of candidate tryptic peptides. By using this approach, we did encounter some problems with partial trypsin cleavage (dipeptide 12) and found that Glu-C failed to digest a peptide that has a Glu near the C-terminus. There is a precedent for both of these findings (40), which is likely the reason for our observed minor variations in peptide maps from experiment to experiment. Nonetheless, we were able to deduce correct sites with little ambiguity, and within a single ^{32}P-labeling experiment, the digestion patterns of

phosphopeptides were highly reproducible. Peptide 9 containing Ser^{345} exhibited heterogeneity by HPLC analysis and produced multiple peptides on cleavage with Asp-N despite the presence of only one phosphorylated site. An analysis of the sequence reveals that this peptide contains two cysteines. Thus, formation of intramolecular or intermolecular disulfide bonds or different oxidative states of cysteine may contribute to the observed heterogeneity. Additionally, we observed that the HPLC peaks in the 7–9 region were more heterogeneous in PR-B than PR-A. Alkaline gel analysis revealed that these HPLC peptides from PR-B may contain additional minor phosphopeptides (36). Because of problems with the loss of peptide 12 after secondary digests, we had to directly analyze this site by amino acid sequencing. Because T47D receptors are trace proteins, we used purified *Baculovirus*-produced PR-B to obtain enough protein for microsequencing. Because phosphopeptides can fractionate with different retention times on HPLC in their phospho and dephospho forms (5), *Baculovirus*-produced receptors are useful only if they are also phosphorylated on the same sites as T47D receptors. In a separate study we have determined that *Baculovirus*-produced hPR in Sf9 insect cells is phosphorylated on all of the same sites as T47D receptors except for peptide 9 (Ser^{345}). Thus recombinant PR is suitable for identification of the site in peptide 12.

Different results have been reported in the literature for the effects of hormone on site specific phosphorylation of steroid receptors. In studies with rabbit PR expressed in COS-1 cells, hormone treatment resulted in a 7-fold increase in net phosphorylation that did not seem to involve phosphorylation of any major new sites (17). Phosphopeptide mapping showed that hormone stimulated a general increase in all phosphopeptides (17). However, identification of sites in rabbit PR was not reported. Similar findings were reported for hER expressed in COS-1 cells. Phosphopeptide mapping did not detect any major new sites on hormone treatment (14). In contrast, estrogen was reported to induce phosphorylation of a major new site at Ser^{167} in endogenously expressed hER in MCF-7 breast cancer cells (16). Recently, enhanced phosphorylation of specific sites in GR in response to hormone treatment were reported (41). Analogous to the present studies, mapping of cPR has clearly shown that hormone induces two new major phosphorylation sites at Ser^{367} (15) and Ser^{530} (5) and at the same time stimulates an increase in two other basal sites (5), and earlier phosphotryptic peptide mapping studies of hPR without site identification also indicated that hormone induced at least one and possibly a second phosphorylation site. The reason for the apparent discrepancies in the literature is not known. This could be due to differences in methods and cell-specific factors or to real differences between classes of steroid hormone receptors, including the same receptor from different species. In this regard, it is of interest to note that the estrogen-inducible site in hER at Ser^{167} was only detected with ER expressed endogenously in MCF-7 cells and not with hER expressed heterologously in COS-1 cells.

It is worth noting that Ser^{102} is the third PR-B-specific site identified thus far in hPR. Numerous studies have shown that the two PR isoforms have distinct functional capabilities. PR-B for example is more active toward the mouse mammary tumor virus enhancer and promoter than synthetic progesterone response elements, whereas PR-A is more active toward the ovalbumin promoter. Even more interesting are recent findings that PR-A has the capability on a promoter where it is a poor activator to act as a dominant transrepressor of other steroid receptors, including PR-B, androgen receptor, GR, mineralocorticoid receptor, and ER. The mechanism for this repressor function is not known but may involve interaction of PR-A with cell- and promoter-specific factors (42, 43). Sartorius *et al.* (43) demonstrated that the N-terminal segment unique to PR-B may contain a transcriptional activation domain separate from activating function-1 (located on the N-terminal side of the DNA-binding domain) and activating function-2, which is in the C-terminus. In addition, they showed that this domain alone is responsible for the band heterogeneity observed on SDS gels for full-length PR-B, suggesting that phosphorylation may be responsible. Our finding of three phosphorylation sites in this region provides support for this notion. These results taken together suggest that phosphorylation of B-specific sites may contribute to the functional activity of this domain. The identification of all the B-specific sites permits the construction of site-directed mutations to evaluate the role of phosphorylation in PR-B-mediated functions critically.

All three hormone-inducible sites, Ser^{102}, Ser^{294}, and Ser^{345}, as well as a previously identified site at Ser^{162}, are in consensus motifs for serine-proline-directed kinases (Ser/Thr-Pro). Most of the sites that have been identified in steroid hormone receptors are also Ser-Pro motifs, suggesting that steroid receptors may be major cellular targets for proline-directed kinases, which include the MAP kinases and cyclin-dependent kinases such as Cdc2 and Cdks (see review, 4). Our preliminary data have shown that Cdk2 *in vitro* efficiently and specifically phosphorylates three basal sites that are also phosphorylated *in vivo*, one of which is Ser^{162}, but it does not phosphorylate the three hormone-inducible sites identified in this study. Therefore, it will be important in future studies to analyze phosphorylation of hPR and its activity toward target genes controlling the cell cycle. CKII consensus sites also seem to be common targets in steroid receptors including GR (7), Ser^{167} of hER (16), VDR (20), and the PR-B-specific site at Ser^{81} (36). This may also be of physiological relevance, because CKII is a nuclear enzyme and has been found to modify sites in a number of other transcription factors (44). Thus, it seems that a minimum of three protein kinases are

involved in modification of hPR, CKII, cyclin-dependent Cdk2 or a related kinase, and an as-yet identified kinase(s) that phosphorylates the hormone-inducible sites.

MATERIALS AND METHODS

Materials

R5020 and carrier-free [^{32}P]H_3PO_4 were obtained from Dupont/New England Nuclear Products (Boston, MA). Protein-A Sepharose was obtained from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Tosylphenylalanyl chloromethyl ketone-treated trypsin was obtained from Worthington Biochemical Corp. (Freehold, NJ). Sequencing grade endoproteinases Asp-N and Glu-C were purchased from Boehringer Mannheim (Indianapolis, IN). Phenylisothiocyanate and HPLC reagents were obtained from J. T. Baker Chemical Corp. (Phillipsburg, NJ). Triethylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and sequencing grade trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO). Sequelon-AA membranes and Mylar sheets were obtained from Millipore Corp. (Milford, MA). AB-52 is a mouse monoclonal immunoglobulin G produced against purified hPR that recognizes both A and B forms of the receptor (45). Minimum essential medium (MEM) was purchased from Irvine (Santa Ana, CA). Phosphate-free MEM was obtained from GIBCO BRL (Grand Island, NY). All other chemicals were reagent grade.

Cell Culture, Metabolic Labeling, and Receptor Preparations

T47D human breast cancer cells were cultured and grown in 75-cm^2 T-flasks for 2 weeks with frequent changes of media as previously described (28). Before harvesting, cells were incubated for 24 h in MEM containing 5% fetal calf serum that has been stripped of steroid hormones by treatment with dextran-coated charcoal. For steady state labeling with [^{32}P]orthophosphate, the serum-containing medium was removed, and cells were preincubated in phosphate-free serum-free medium for 1 h at 37 C. Cells were then incubated for 6 h at 37 C in phosphate-free MEM containing [^{32}P]orthophosphate (0.83 mCi/ml). Cells were treated with 40 nM R5020 for the times indicated in the figure legends.

Cells were harvested using 1 mM EDTA in Earle's balanced salt solution and homogenized at 4 C in a Teflon-glass Potter-Elvehjem homogenizer (Fisher, Pittsburgh, PA) in KPFM buffer [50 mM potassium phosphate (pH 7.4), 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 12 mM monothioglycerol] containing 0.5 M NaCl and a mixture of proteinase inhibitors as previously described (29). The homogenates were centrifuged at 100,000 × *g* for 30 min, and the supernatant (whole cell extract) was dialyzed against KPFM to remove the majority of the salt before the immunoprecipitation step.

Immunoprecipitation and Gel Purification of PR

Protein A Sepharose bound with the specific monoclonal antibody, AB-52, was prepared as previously described (29). Dialyzed whole-cell extracts containing PR were incubated with AB-52-coated Protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden) on an end-over-end rotator for 4 h at 4 C. To remove nonspecific proteins, Protein A Sepharose was washed at least three times by centrifugation in buffer containing 0.3 M NaCl. Bound receptors were then eluted with 2% SDS sample buffer and electrophoresed on a 7.0% discontinuous SDS polyacrylamide gels as previously described (29). ^{32}P-Labeled receptors were located by autoradiography of wet gels, and the gel pieces corresponding to the PR-A and PR-B were excised and counted for radioactivity incorporated by Cerenkov counting.

HPLC Analysis of Trypsin-Digested PR

SDS gel slices containing PR were placed in 1.5-ml microfuge tubes, washed at room temperature with 50% methanol for 1 h, with H_2O for 30 min, and with 50 mM ammonium bicarbonate for 5 min. Trypsin digestion was carried out by adding 20 μg trypsin and 500 μl 50 mM ammonium bicarbonate to the tubes containing gel slices. The tubes were placed in a 37 C water bath, and four additional aliquots of trypsin were added at 4-h intervals. Trypsin cleaves on the carboxyl-terminal side of Lys and Arg residues, but Lys-Pro and Arg-Pro bonds are resistant, and the presence of a negatively charged residue adjacent to the basic residue greatly decreases the hydrolysis rate. Tryptic phosphopeptides were dried in Speedvac (Savant Instruments, Hicksville, NY), dissolved in 50% formic acid, applied to a Vydac (Hesperia, CA) C18 reverse-phase column in 0.1% TFA in water, run at a flow rate of 1 ml/min, and eluted with a linear gradient from 0–45% acetonitrile over 90 min. ^{32}P-Labeled peptides were identified with an on-line model IC Flo-One β-radioactivity flow detector (Radiomatic Instruments, Inc., Tampa, FL) (5).

Characterization of Tryptic Phosphopeptides by Proteinase Digestion and Manual ^{32}P Release

After the fractions containing tryptic phosphopeptides resolved by reverse-phase HPLC were collected, they were further purified by electrophoresis on a 40% alkaline polyacrylamide gel (39). The gel was dried and autoradiographed, and bands containing tryptic phosphopeptides were excised and eluted with H_2O overnight. Eluted peptides were dried in a Speedvac and subsequently subjected to digestion with the endoproteinases Glu-C and Asp-N. Glu-C cuts on the C-terminal side of Glu, except for Glu-Pro bonds. In addition, Glu-X bonds within three residues of the end of a peptide are cleaved poorly (40). Asp-N cuts on the N-terminal side of Asp residues. Peptides, digested and undigested, were analyzed by peptide gel electrophoresis or manual Edman degradation. Routinely, peptides to be digested with Asp-N were dissolved in 200 μl 50 mM sodium phosphate buffer, pH 8, containing 0.2 μg Asp-N and incubated at 37 C for 4 h. Glu-C digestion was performed using 1 μg Glu-C in 200 μl 25 mM ammonium bicarbonate, pH 7.8, for 8 h at 37 C.

To localize the position of phosphoamino acids in the peptides, manual Edman degradation described by Sullivan and Wong (46) was used. Briefly, the peptide to be analyzed was dissolved in 30 μl 50% acetonitrile and spotted on an arylamine-Sequelon disc, which was placed on a Mylar sheet on top of a heating block set at 50 C. After 5 min, the aqueous solvent was evaporated, and the disc was removed from the heating block. Five microliters of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide solution (10 mg/ml in 2-[N-morpholino]-ethanesulfonic acid, pH 5.0) was added to the disc to allow the peptide to covalently link to the disc, and the disc was placed at room temperature for 30 min. The disc was then washed five times with water and five times with TFA to remove unbound peptide. The disc was then washed three times with methanol and subjected to Edman degradation. The disc was treated at 50 C for 10 min with 0.5 ml coupling reagent (methanol, water, triethylamine, and phenylisothiocyanate; 7:1:1:1, vol/vol). After five washes with 1 ml methanol, the disc was treated at 50 C for 6 min with 0.5 ml TFA to cleave the amino-terminal amino acid. The TFA solution was placed in a scintillation vial, and the disc was washed with 1 ml TFA and 42.5% phosphoric acid (9:1, vol/vol). The wash was combined with the TFA solution, and the released

[^{32}P] was determined by Cerenkov counting. At this stage, the disc can be either stored in methanol at −20 C or washed five times with 1 ml methanol before the next cycle is started.

Purification of *Baculovirus*-Expressed hPR-B

Human PR-B used in protein-sequencing experiments was produced as a full-length recombinant protein from the *Baculovirus* expression system as previously described (45, 47). It was purified to apparent homogeneity by immunoaffinity chromatography. R5020 was added during the last 4 h of infection of the *Spodaptera frugiperda* (Sf9) cells with the PR-B-expressing recombinant virus. Thus, purified PR used in these experiments was bound to hormone. Prior studies showed that *Baculovirus*-produced hPR is functionally and structurally indistinguishable from hPR synthesized endogenously in mammalian cells (48).

Preparation and Isolation of Peptides for Sequencing

Purified *Baculovirus*-expressed PR-B was digested with trypsin (5% wt/wt). Tryptic peptides were resolved by reverse-phase HPLC as described earlier. Fractions with retention times corresponding to the ^{32}P-labeled tryptic phosphopeptides from T47D cells were collected, dried in a Speedvac, and sequenced using an automated sequencer (5).

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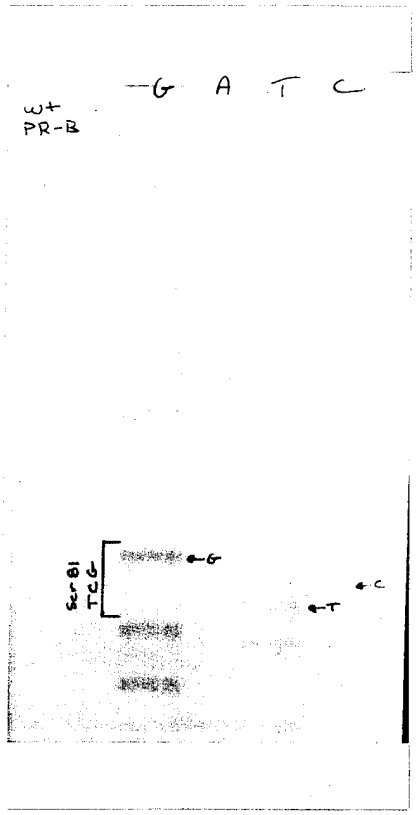
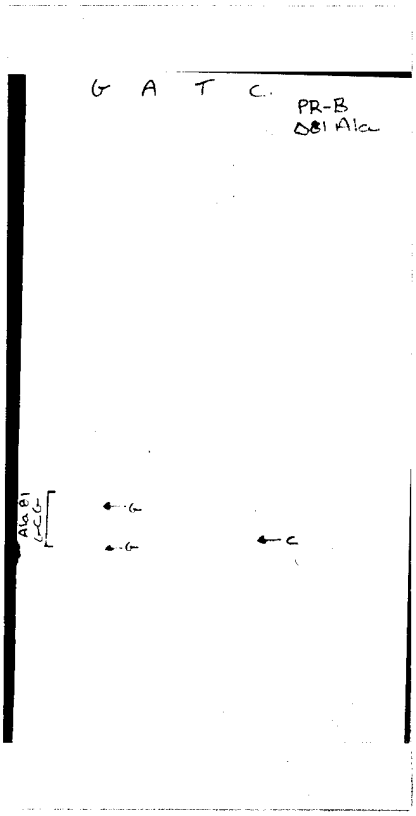
Appendix B: Seqence analysis of mutant Ala[81]





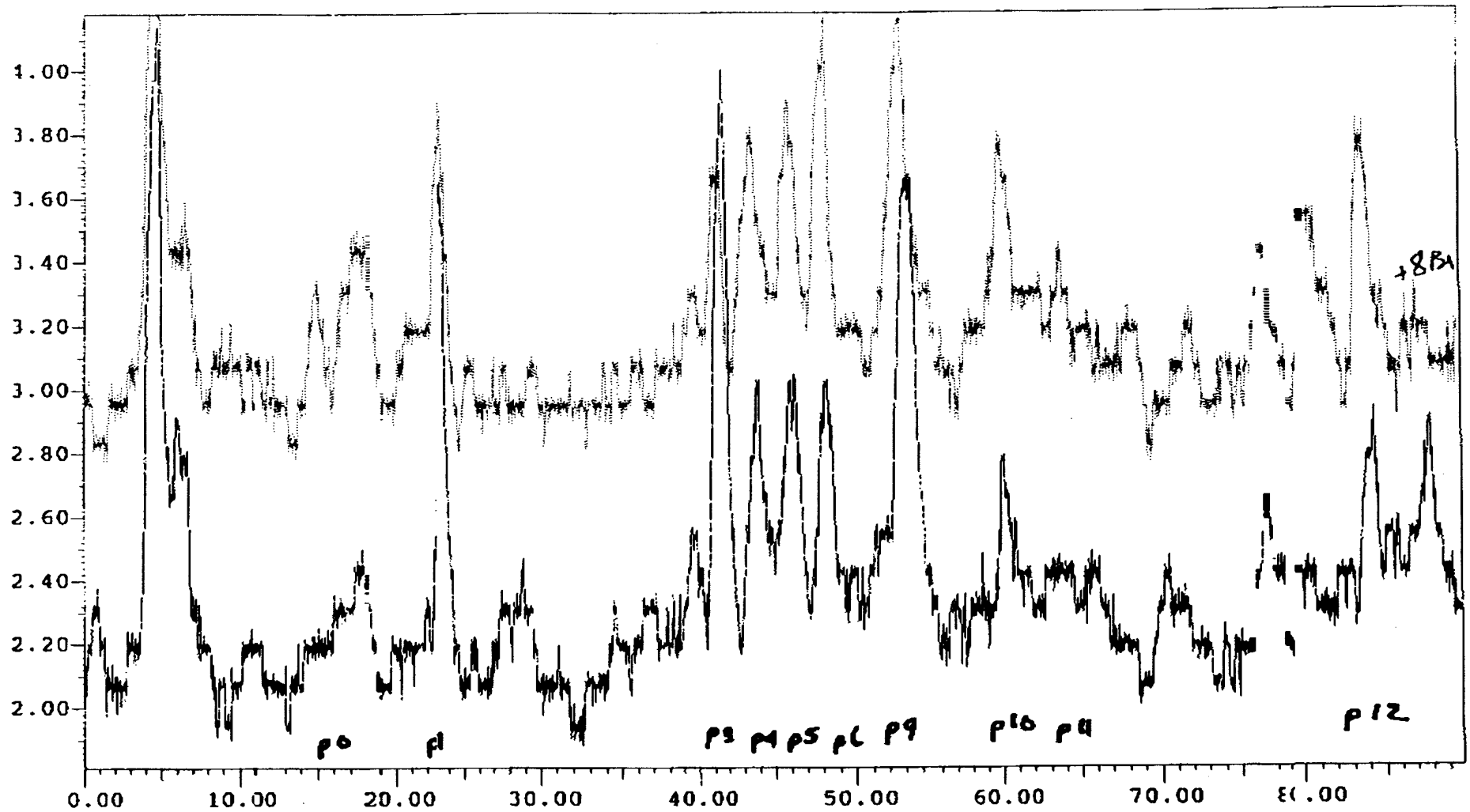
WT

TCG(Serine)

Mutant

GCG(Ala)

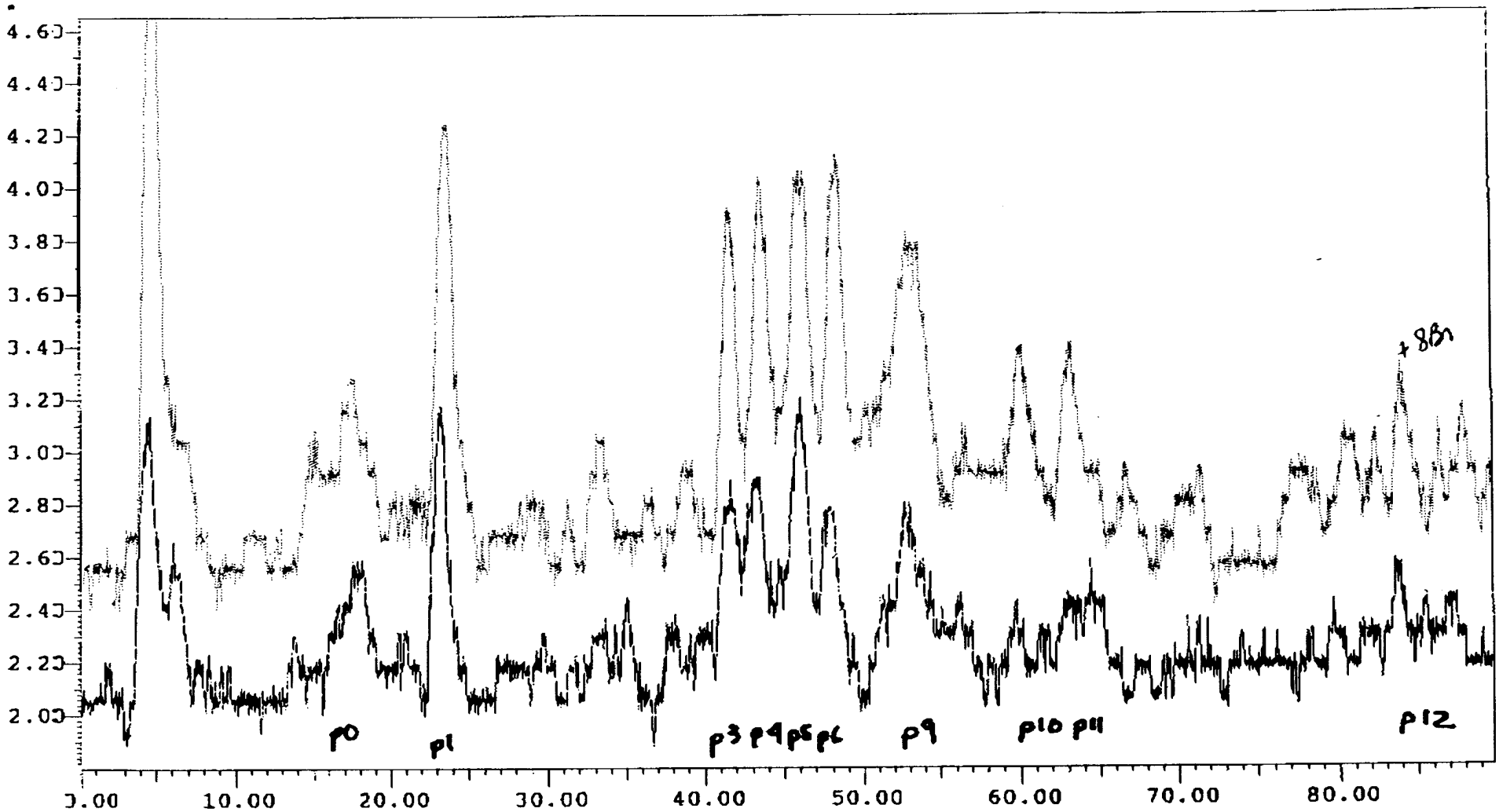
Appendix C: Phosphotriptic peptide maps of hPR phosphorylated in the presence of R5020, RU486 and RU486 plus 8 Br cAMP. This experiment was done in collaboration with Dr. Candy Beck. The exact retention time of each phosphopeptide is different from that of in Fig. 1 due to the use of her HPLC equipped with a C18 column. The HPLC profiles of R5020 and R5020 plus 8-Br cAMP are superimposed, so are the RU486 and RU486 plus 8 Br cAMP, to see if there is any significant difference between different treatments.



UV_PPA.132\DAT001.DTI 32P cpm
5B

UV_PPA.133\DAT001.DTI 32P cpm
6B

CB340
R5020
R5020 + 8B1



UV_PPA.109\DAT001.DTI 32P cpm
3E

UV_PPA.110\DAT001.DTI 32P cpm
4E

CB329
RU
RU + 8Br